Zootaxa 1484: 1–38 (2007)
www.mapress.com/zootaxa/

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

Molecular systematics of Serrasalmidae: Deciphering the identities of piranha species and unraveling their evolutionary histories

BARBIE FREEMAN[1], LEO G. NICO[2], MATTHEW OSENTOSKI[1], HOWARD L. JELKS[2] & TIMOTHY M. COLLINS[1,3]

[1]*Dept. of Biological Sciences, Florida International University, University Park, Miami, FL 33199, USA.*
[2]*United States Geological Survey, 7920 NW 71st St., Gainesville, FL 32653, USA. E-mail: Leo_Nico@usgs.gov*
[3]*Corresponding author. E-mail: CollinsT@fiu.edu*

Table of contents

Abstract 1
Introduction 2
Overview of piranha diversity and systematics 3
Material and methods 10
Results 16
Discussion 16
Conclusions 26
Acknowledgments 26
References 26
Appendix 1. Photographs of living and preserved specimens 30
Appendix 2. GenBank sequences used in this study. 38

Abstract

Piranhas and their relatives have proven to be a challenging group from a systematic perspective, with difficulties in identification of species, linking of juveniles to adults, diagnosis of genera, and recognition of higher-level clades. In this study we add new molecular data consisting of three mitochondrial regions for museum vouchered and photo-documented representatives of the Serrasalmidae. These are combined with existing serrasalmid sequences in GenBank to address species and higher-level questions within the piranhas using parsimony and Bayesian methods. We found robust support for the monophyly of *Serrasalmus manueli*, but not for *Serrasalmus gouldingi* when GenBank specimens identified as *S. gouldingi* were included in the analysis. "*Serrasalmus gouldingi*" sequences in GenBank may, however, be misidentified. Linking of juveniles to adults of the same species was greatly facilitated by the addition of sequence data. Based on our sampling and identifications, our data robustly reject the monophyly of the genera *Serrasalmus* and *Pristobrycon*. We found evidence for a well-supported clade comprised of *Serrasalmus*, *Pygocentrus*, and *Pristobrycon* (in part). This clade was robustly supported in separate and combined analyses of gene regions, and was also supported by a unique molecular character, the loss of a tandem repeat in the control region. Analysis of specimens and a literature review suggest this clade is also characterized by the presence of a pre-anal spine and ectopterygoid teeth. A persistent polytomy at the base of this clade was dated using an independent calibration as 1.8 million years old, corresponding to the beginning of the Pleistocene Epoch, and suggesting an origin for this clade more recent than dates cited in the recent literature. The sister group to this clade is also robustly supported, and consists of *Catoprion*, *Pygopristis*, and *Pristobrycon striolatus*. If the term piranha is to refer to a monophyletic clade, it should be restricted to *Serrasalmus*, *Pygocentrus*, and *Pristobrycon* (in part), or expanded to include these taxa plus *Pygopristis*, *Catoprion*, and *Pristobrycon striolatus*.

Accepted by Gilbert: 31 Mar. 2007; published: 28 May 2007

Key words: Serrasalmidae, piranhas, Characidae, Characiformes, *Serrasalmus*, *Pristobrycon,* phylogeny, polytomy, biogeography

Introduction

Piranhas are neotropical freshwater fishes belonging to the order Characiformes and infamous for their formidable dentition and predatory habits. According to Machado-Allison (1983, 1985) they comprise a monophyletic group within the Serrasalminae, a subfamily of the Characidae that also includes the pacus. Some recent researchers still maintain the subfamily (Jégu 2003), while others recognize piranhas and their relatives as a distinct family, the Serrasalmidae (Géry 1972, 1977; Reis 1998; Calcagnotto *et al.* 2005). In this manuscript, we treat piranhas and pacus as a separate family, Serrasalmidae, realizing the exact relationship of this group to other characiformes is yet to be determined.

Serrasalmids are endemic to South America, with species distributed in all major and some minor Atlantic river systems from about 10° N latitude south to about 35° S latitude. Many members of the family are in demand as aquarium ornamentals, and several pacus (e.g., *Piaractus* and *Colossoma*) are economically important to commercial fisheries and aquaculture (Araujo-Lima & Goulding 1997; Castagnoli 2000). Piranhas are generally less valued, although they are commonly consumed by subsistence fishers and frequently sold for food in local markets. A few piranha species occasionally appear in the aquarium trade, and, in recent decades, dried specimens have been marketed as tourist souvenirs (L. G. Nico, pers. obs.). Piranhas occasionally bite and sometimes injure bathers and swimmers, but truly serious attacks are rare and the threat to humans has been largely exaggerated (Braga 1975; Goulding 1980; Nico & Taphorn 1986; Sazima & Andrade-Guimaraes 1987; Haddad & Sazima 2003). However, piranhas are a considerable nuisance to commercial and sport fishers because they steal bait, mutilate catch, damage nets and other gear, and may bite when handled (Agostinho *et al.* 1997; L. G. Nico, pers. obs.).

Ecologically, piranhas are important components of their native environments. Although largely restricted to lowland drainages, these fishes are widespread and inhabit diverse habitats within both lotic and lentic environments. Some piranha species are abundant locally and multiple species often occur together. For example, as many as seven different piranha species have been recorded from a single reach of Caño Maporal, a small savannah stream in Venezuela (Nico & Taphorn 1988; L. G. Nico, pers. obs.). As both predators and scavengers, piranhas influence the local distribution and composition of fish assemblages (Nico & Taphorn 1988; Winemiller 1989; Sazima & Machado 1990). In spite of the group's reputation as carnivores, certain piranha species consume large quantities of seeds, but unlike *Colossoma* and *Piaractus*, herbivorous piranhas thoroughly masticate and destroy all seeds eaten and consequently do not function as dispersers (Goulding 1980; Nico 1991).

The taxonomy and systematics of piranhas and their relatives are complicated and much remains unsettled. Consequently, both species identification and phylogenetic placement of many taxa are problematic. Both nomenclatural uncertainty and difficulties involving positive identification have long frustrated scientists and hampered systematic as well as non-taxonomic research (e.g., ecology and physiology) on piranhas. For instance, investigators have misidentified study animals as a single species when two or more species were actually represented, or have erroneously recognized several species in a sample that actually consisted of a single species. As a result, names used for piranhas in the literature, field survey reports, museum collections, and other sources (e.g., GenBank) are often suspect. Sensitive to this issue, a number of ecologists have been reluctant to assign names, using codes rather than providing species or even generic names of questionable validity (e.g., Goulding *et al*. 1988).

Some reasons for the continued confusion include: 1) shortage of comparative material; 2) lack of distinct or reliable external features useful in distinguishing most genera as well as many species; 3) wide intra-specific variation in morphology (generally related to ontogenetic changes); 4) wide intra-specific variation in

color pattern in both preserved specimens and live individuals within members of a population, and between geographic localities (often associated with ontogeny, reproductive condition, or environmental influences); 5) marked overlap in color patterns and morphologies among different species; 6) poorly known geographic ranges; 7) a number of poorly-defined nominal species (in some cases coupled with the absence or loss of type material); 8) probable existence of species complexes (e.g., *Serrasalmus rhombeus*); 9) uncertainty about the generic placement of certain species in *Serrasalmus* versus *Pristobrycon*; and 10) the existence of numerous synonymies, some speculative (e.g., Fink 1993; Machado-Allison & Fink 1996; Machado-Allison 2002; Jégu 2003). Over the past few decades, several new species have been described. Although some are markedly distinct (e.g., *Pristobrycon maculipinnis* Fink & Machado-Allison 1992) most of these recent additions closely resemble previously described nominal species (e.g., *S. altuvei* Ramirez 1965, followed by *S. compressus* Jégu *et al*. 1991, and *S. hastatus* Fink & Machado-Allison 2001).

The goal of our research was to use mitochondrial DNA sequences to elucidate serrasalmid relationships and to aid in species-level identification. To resolve systematic questions, we analyzed three mitochondrial gene regions with varying rates of evolution: the relatively slowly evolving small (12S) and large (16S) ribosomal RNA (rRNA) genes, along with the more rapidly-evolving control region. We focused on the species *S. manueli*, *S. gouldingi*, and an unidentified species (*Serrasalmus* sp. "A"). Because of the difficulty in identifying juvenile and adult specimens that were either fresh or preserved, genetic analyses were necessary to confirm field identifications, to determine whether the three piranhas were distinct, and to clarify their systematic relationships. We have included photographs to document similarities and differences in fresh and preserved vouchered specimens. An additional objective was to investigate the issue of monophyly among certain piranha genera, in particular, *Serrasalmus* and *Pristobrycon*. To test hypotheses about a persistent polytomy within the piranhas, we expanded our sampling to include 12S, 16S, and control region sequences available for Serrasalmidae in GenBank. Finally, because of the confusion surrounding serrasalmid systematics and taxonomy, we discuss the diversity of the piranhas and provide a brief overview of past research on their systematics.

Overview of piranha diversity and systematics

Diversity within the Serrasalmidae. Compared to many neotropical fish groups, the Serrasalmidae is well defined, and there is wide agreement concerning which genera and species should be included (Machado-Allison 1983; Jégu 2003). Serrasalmids are medium to large-sized characids (up to about 1-m long) generally characterized by a deep, laterally compressed body with a series of mid-ventral abdominal spines or scutes, and a long dorsal fin (>16 rays). Most species also possess an anteriorly-directed spine just before the dorsal fin extending from a supraneural bone; exceptions include members of the genera *Colossoma*, *Piaractus*, and *Mylossoma* (Jégu 2003). Jégu (2003) reported the family as comprised of 15 genera and 80 valid species, although he noted that the status of as many as eight of these "valid" forms was uncertain (*incertae sedis*). According to Jégu, in addition to the four piranha genera, the family currently includes *Acnodon* (3 species), *Catoprion* (1), *Colossoma* (1), *Metynnis* (11), *Mylesinus* (3), *Myleus* (14 or 15), *Mylossoma* (3), *Ossubtus* (1), *Piaractus* (2), *Tometes* (2), and *Utiaritichthys* (2).

Traditionally, "piranhas" or "true piranhas" are a group that includes only the four genera *Serrasalmus*, *Pristobrycon*, *Pygocentrus*, and *Pygopristis*. These genera possess a number of morphological traits that separate them from other serrasalmids (Machado-Allison 1985; Machado-Allison & Fink 1996; Jégu 2003). In particular, they are most easily distinguished by their unusual dentition. All piranhas have a single row of sharp teeth in both jaws; the teeth are tightly packed and interlocking (via small cusps) and used for rapid puncture and shearing. Individual teeth are typically broadly triangular, pointed, and blade-like (i.e., flat in profile). There is minor variation in the number of cusps; in most species the teeth are tricuspid with a larger

middle cusp that makes the individual teeth appear markedly triangular. The exception is *Pygopristis* which has pentacuspid teeth and a middle cusp that is usually only slightly larger than the other cusps (Machado-Allison 1982, 1985). A few authors apply the term "piranha" or "true piranhas" more broadly, grouping them with what is considered to be their closest relatives, the scale-eating, monotypic genus *Catoprion* and the plant-eating genus *Metynnis* (Machado-Allison 1982, 1985; Ortí *et al*. 1996; Nakayama *et al*. 2002). However, the shape of the teeth of *Catoprion* and *Metynnis* are markedly different from that of the four traditional piranha genera, and, similar to other serrasalmids, their premaxillary teeth are in two rows, not one (Machado-Allison & Fink 1995, 1996).

The number of piranha species is not known, and new species continue to be described. Fink (1988) stated that fewer than half of the approximately 60 nominal species of piranhas were valid. In a more recent treatment, Jégu (2003) recognized a total of 38 or 39 species, although the validity of some taxa remains questionable. Based on the annotated list of Jégu, the most species rich genus is *Serrasalmus* (24 species, perhaps as many as 28), followed by *Pristobrycon* (5), *Pygocentrus* (3, possibly 4), and *Pygopristis* (1). The natural distribution of many piranha species is poorly known. According to Jégu (2003), 25 species are distributed in the Amazon basin, 16 in the Orinoco, 9 in rivers of the Guyanas, 3 in the Paraguay-Paraná, and only 2 in the São Francisco. Some species have extremely broad geographic ranges, occurring in more than one of the major basins mentioned above, whereas others appear to have much more limited distributions.

Systematic relationships. Investigators interested in the systematics of piranhas and their relatives have used a variety of methods to evaluate and compare a broad range of characters. Early studies focused on morphology (e.g., Eigenmann 1915; Norman 1929; Gosline 1951; Machado-Allison 1982, 1985) while a few of the more recent investigations have dealt with karyological (Nakayama *et al*. 2002, and references therein) and parasitological (Van Every & Kritsky 1992) characters. Some researchers made minor attempts to compare piranha behavioral ecology (e.g., Nico 1991). During the past decade, investigations have included analysis of molecular data (Ortí *et al*. 1996; Calcagnotto *et al*. 2005; Hubert *et al*. 2006). While most of the studies agree the family Serrasalmidae is monophyletic, there is disagreement concerning relationships within this family.

As reviewed in detail by Machado-Allison (1985, 2002), early morphological studies involved numerous changes in both the number and hierarchy of serrasalmid genera. For example, Eigenmann (1915) recognized six piranha genera (*Pygopristis*, *Gastropristis*, *Rooseveltiella*, *Pristobrycon*, and *Serrasalmo*) whereas Norman (1929) and Gosline (1951) relegated all species to two genera (*Serrasalmus* and *Pygopristis*). In contrast, Géry (1972, 1976) grouped all piranhas into a single genus, *Serrasalmus,* dividing the species among five subgenera. The first cladistic analysis of the Serrasalminae was conducted by Machado-Allison (1982, 1983, 1985). Based on a comparative study of 65 anatomical characters, mostly osteological and myological, he divided 13 serrasalmid genera into two major clades, with the piranha genera monophyletic (Fig. 1A). Largely limiting himself to generic-level analyses, Machado-Allison's phylogenetic hypothesis endured unchallenged for many years, consequently, his tree of generic relationships has been widely cited and often reprinted (e.g., Machado-Allison 1983, 1985, 2002; Lundberg *et al*. 1986; Machado-Allison & Fink 1996). As part of a later review focusing on the genus *Pristobrycon*, Machado-Allison *et al*. (1989) offered a slightly modified hypothesis, revising their piranha clade so as to reflect possible evolution of piranhas based, in part, on the presence versus absence of the pre-anal spine (Fig. 1B).

Morphometric techniques have also been used to address serrasalmid relationships and to quantify differences among taxa more precisely (Géry 1972; Fink 1989; Machado-Allison *et al*. 1989; Fink & Zelditch 1995, 1997). These methods have proven valuable for quantifying variation in shape but have been less useful for clarifying serrasalmid relationships. For example, Fink (1993) determined that head width dimensions separated *Pygocentrus* from other piranhas but was unable to detect any significant shape differences among the three recognized species of *Pygocentrus*.

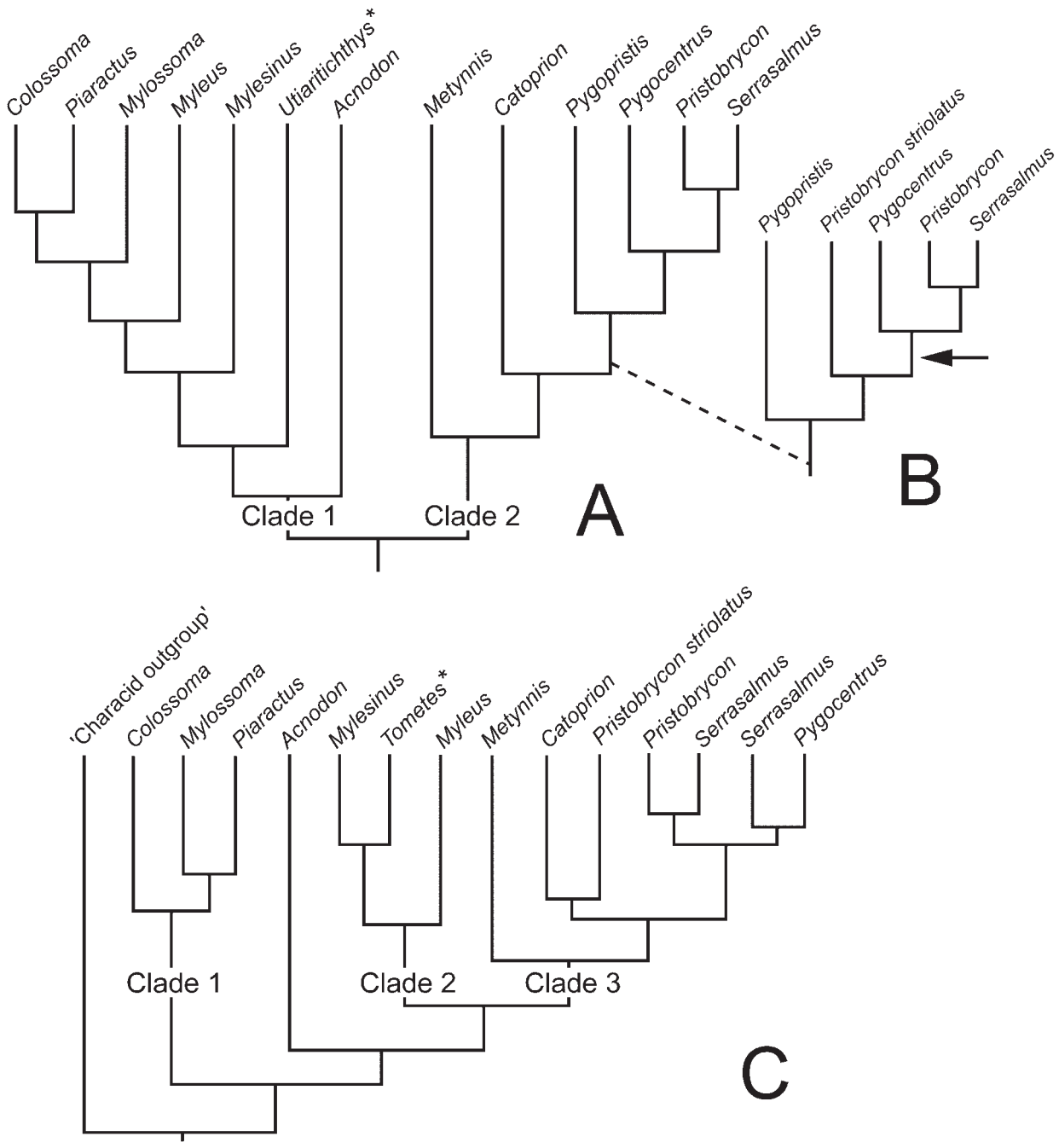

FIGURE 1. Alternative hypotheses of serrasalmid relationships: (A) Machado-Allison (1983, 1985), based on morphology, divides family into two major clades; (B) Machado-Allison *et al.* (1989) revised piranha clade showing the position of *Pristobrycon striolatus* if absence of pre-anal spine is considered to be primitive character (arrow indicates occurrence of this trait); (C) Ortí *et al.* (1996), based on mitochondrial ribosomal RNA sequence data, defines three major clades. Upper tree includes 13 of the currently 15 recognized genera, lower tree includes 11 genera. Note: the genus *Tometes* was presented in original tree of Ortí *et al.* (1996) as "N. gen. A" (P. Petry, *pers. comm.* 2005). The authors also stated that specimens assigned by Machado-Allison (1982, 1983) to *Utiaritichthys* do not belong to that genus, apparently suggesting the specimens are *Tometes*.

The first published work on serrasalmid relationships using sequence data is Ortí *et al*. (1996) who performed a cladistic analysis using mitochondrial rRNA genes to test Machado-Allison's 1982 phylogeny. The results from the molecular data differed from that based on morphological data, suggesting the family was divided into three groups rather than the two proposed by Machado-Allison (Fig. 1C). Moreover, their analysis indicated that *Serrasalmus*, *Pristobrycon*, and *Myleus* may be para- or polyphyletic. Ortí *et al.* (1996) also

reported that mitochondrial data showed *Pristobrycon striolatus* to be very different from the other *Pristobrycon* analyzed in their study. An additional unpublished molecular study by Ortí *et al.* (2000) based either on 12S and 16S, or control region sequence data, included trees that contain several species that are not monophyletic. They also reported that the genera *Serrasalmus* and *Pristobrycon* were paraphyletic and fell within a clade that included *Pygocentrus*, with a sister clade of *Catoprion*, *Pygopristis* and *Pristobrycon.* Recently, Calcagnatto *et al.* (2005) investigated characiform phylogeny by parsimony analysis of four nuclear and two mitochondrial genes. Focusing on generic and higher-level relationships, their results were inconsistent with those of both Machado-Allison (1983) and Ortí *et al*. (1996), especially in terms of placement of *Colossoma*, *Mylossoma*, and *Piaractus*. Partly because their study included only eight of the 15 serrasalmid genera, Calcagnatto *et al.* (2005) noted that their scheme of relationships within the family should be interpreted with caution. Hubert *et al.* (2006) examined nuclear DNA of sympatric *Serrasalmus* from the Bolivian Amazon, finding evidence of reproductive isolation in some species.

A few recent authors have reported on chromosome numbers and position of nucleolar organizing regions (NORs) of various serrasalmid fishes (e.g., Gaviria *et al.* 2005; Nirchio *et al.* 2005). Ortí *et al.* (1996) mapped the available information (Oliveira *et al.* 1988; Porto *et al.* 1991, 1992; and Cestari & Galetti 1992) onto their phylogenetic tree and concluded there is an obvious trend for chromosome numbers to increase during the evolution of serrasalmids, with the 2*n* number ranging anywhere from 54 to 62 among genera and species sampled. Most piranhas and closely related genera were characterized by having 2*n* = 60, except for *Metynnis* which had 2*n* = 62, and according to Nakayama *et al.* (2001), *Pristobrycon striolatus*, *Catoprion*, and *Pygopristis*, which also possessed 2n = 62. In addition, they reported the autapomorphic reduction of chromosome number (to 2*n* = 58) of one *Serrasalmus* species. A recent study by Gaviria *et al.* (2005) on the karyotype and NORs of *Pygocentrus cariba*, however, does provide additional support for the hypothesized close relationship between *Pygocentrus* and *Serrasalmus*.

Parasitological research intended to obtain a better understanding of serrasalmid relationships is still in its early stages. Van Every and Kritsky (1992) described 13 new species of helminth gill-parasites of the genus *Anacanthorus* taken from central Amazonian piranhas. Included were 10 parasite species from 3 piranha genera (*Pygocentrus*, *Pristobrycon*, and *Serrasalmus*). Using the parasites as indicators of host evolution, the two researchers proposed their own phylogenetic hypothesis (Fig. 2). According to Van Every and Kritsky, the relationships among the three genera evident in their parasite-host cladogram generally supported those offered by Machado-Allison (1983). Similar to the mitochondrial DNA data of Ortí *et al*. (1996), their parasite-host hypothesis suggested that *Pristobrycon* and *Serrasalmus* were paraphyletic. Subsequently, Ortí *et al.* (1996), reanalyzed Van Every and Kritsky's (1992) data set and were able to match the parasite information with their own molecular-based cladogram. They concluded that more information was needed to resolve relationships confidently among the parasites studied. In a subsequent paper, Nakayama *et al.* (2001) noted that differences in parasite species supported recognition of a cryptic species of piranha within *Serrasalmus rhombeus*. Unfortunately, use of parasites to generate phylogenies is problematic (Lovejoy 1997). Moreover, this type of research requires exhaustive sampling and considerable amounts of data to avoid the false conclusion that certain parasites are absent from particular host taxa.

Researchers have long been interested in the diets of serrasalmid fishes and several investigators have attempted to infer or refine serrasalmid systematic relationships by comparing trophic variation (e.g., Géry 1977; Fink 1989; Nico 1991). As a group, the diets of serrasalmid fishes are extremely broad and include seeds, fruits, leaves, various invertebrate and vertebrate prey, as well as fish flesh, scales, and fins. To emphasize the polarity of diets, authors commonly highlight the fruit- and leaf-eating pacus such as *Piaractus brachypomus* and the highly carnivorous piranhas such as *Pygocentrus nattereri*. Most non-piranhas in the family are primarily herbivorous. In contrast, it was long believed that piranhas were strict carnivores. Consequently, Géry (1977) argued that feeding specializations could be used to divide "Serrasalmidae" into three supposedly natural groups or subfamilies: 1) Myleinae (pacus and their allies) composed of vegetarians, 2) Serrasalminae (piranhas) consisting of carnivores, and 3) Catoprioninae composed of the scale eating *Catoprion mento*.

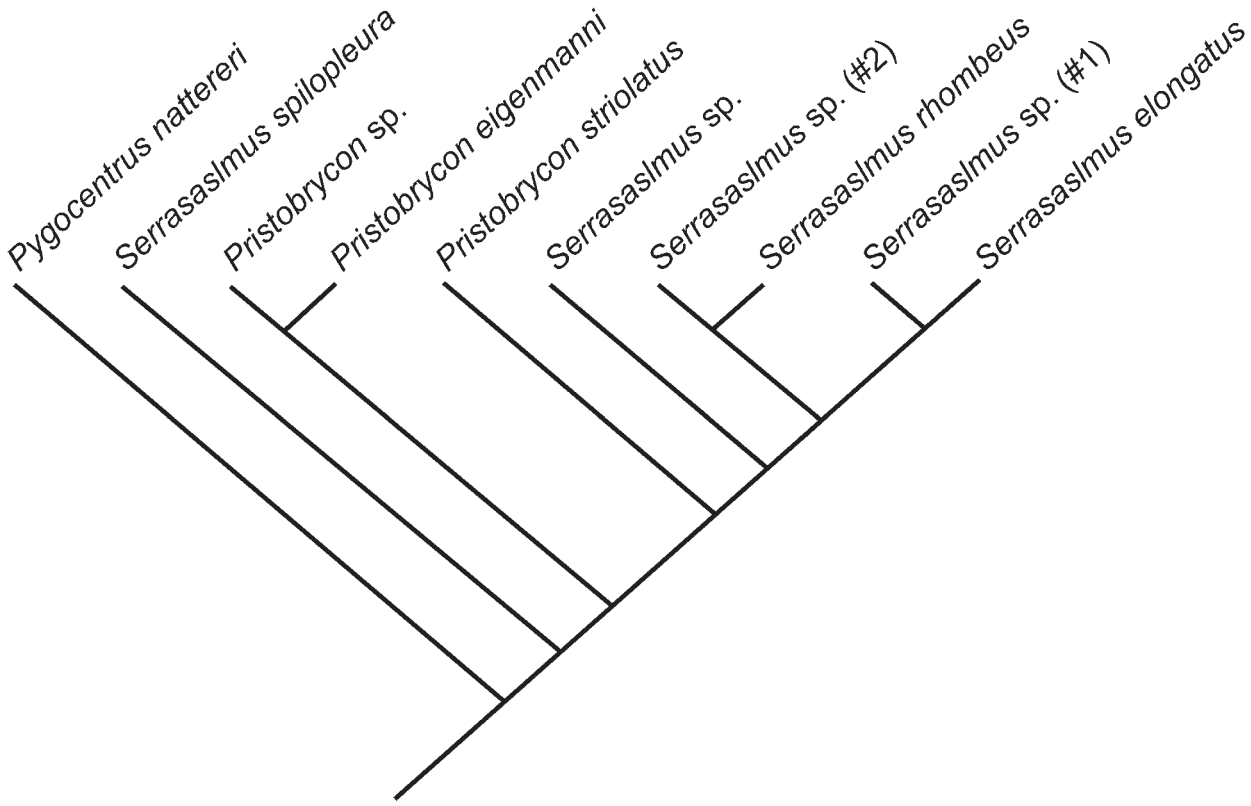

FIGURE 2. Van Every and Kritsky (1992) hypothesis of the evolutionary relationships of 10 piranha species from the central Amazon based on their helminth (*Anacanthorus*) parasite fauna.

Subsequent diet studies of more piranha species exposed flaws in Géry's somewhat simplistic classification scheme, revealing that the diets of most genera and many serrasalmid species are much more complex than previously believed, and not easily divided into trophic specialties (Goulding 1980; Nico & Taphorn 1988; Nico 1991). For instance, many species are known to adopt multiple diets depending on age and resource availability. Nico (1991) attempted to integrate the trophic ecology and piranha phylogenies by using diet and intestine length data for nine serrasalmid genera and superimposing this information onto Machado-Allison's (1985) suggested phylogeny (Fig. 3). Results were mixed, largely because of the variation in trophic characters found for some upper Orinoco species. Based on the variation and complexity of feeding behaviors among serrasalmids, diets alone appear to be of limited use in predicting the phylogenetic relationships among serrasalmid fishes.

The fossil record available for serrasalmid fishes, particularly for piranhas, is relatively sparse. Most known serrasalmid fossils are from the Miocene, although a few unidentified forms are considered Paleocene and two reportedly date to as early as the Late Cretaceous (Arratia & Cione 1996). Miocene remains include those of an unidentified *Serrasalmus* from Peru (Arratia & Cione 1996), a *Colossoma*-like fish unearthed in Colombia (Lundberg *et al*. 1986), and an unidentified non-piranha serrasalmid found in Chile (Rubilar 1994). Reis (1998:359) applied the known fossil evidence to the cladogram of Machado-Allison (1983) and concluded that all serrasalmid genera had originated by the middle Miocene, with the possible exception of three of the four piranha genera (*Pygocentrus*, *Pristobrycon*, and *Serrasalmus*).

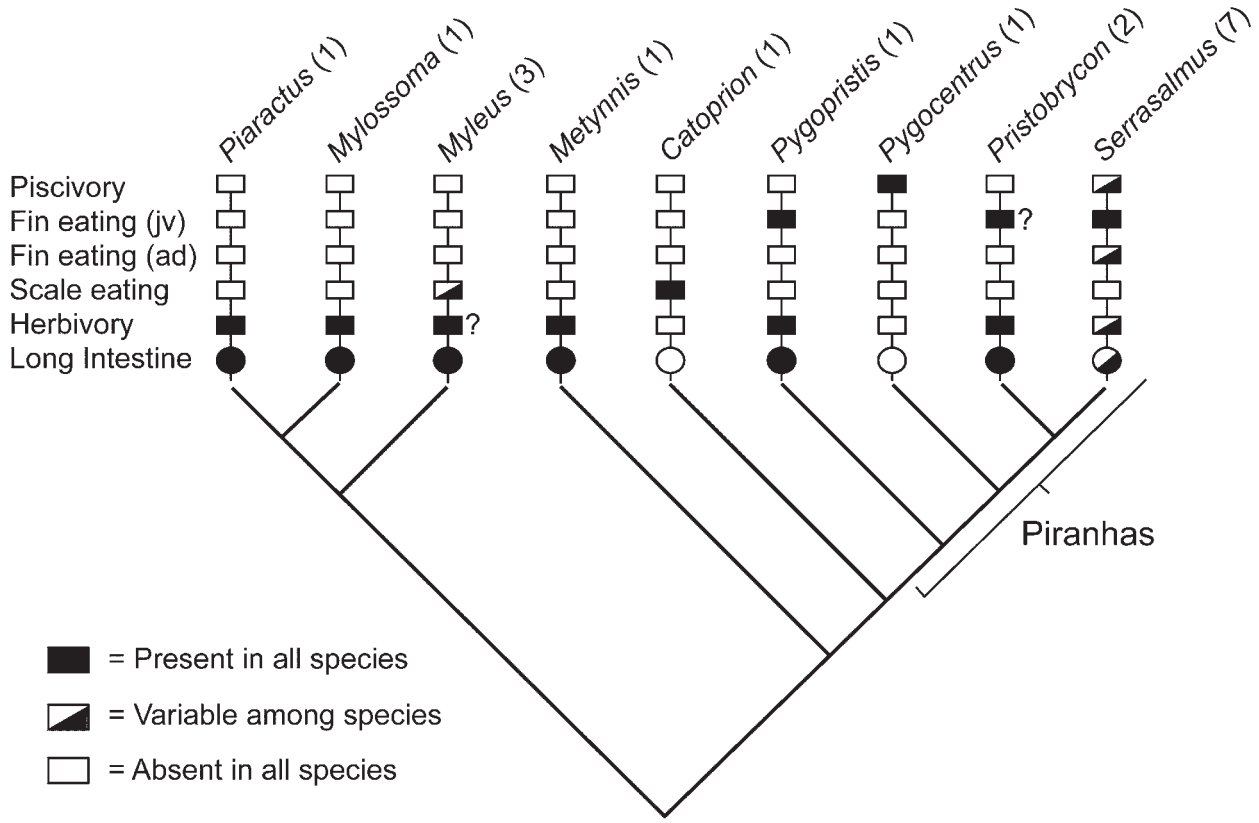

FIGURE 3. Diet and intestinal length data mapped onto Machado-Allison's (1985) proposed phylogeny (modified from Nico 1991). Diet data based on 18 serrasalmid species from the Orinoco River basin (Venezuela); number in parentheses following generic name represents numbers of species in each genus included in study; Jv = juvenile trait; ad = adult trait; long intestine defined as mean intestine length >1.2 X standard length.

Piranhas of southern Venezuela. The piranha fauna of Venezuela is relatively well known, with 16 species appearing in the identification guide by Machado-Allison and Fink (1996, also see Machado-Allison 2002). Nevertheless, many parts of the country are poorly sampled and a few undescribed forms are not included. Little or no information (e.g., diagnostic characters) on juveniles is provided for most piranhas. Consequently, even in Venezuela, there are still taxonomic and identification issues that need to be resolved. Least is known about the piranhas occurring in southern Venezuela, a biogeographically important region that includes the upper Orinoco, upper Negro, and Casiquiare river systems.

The two most diverse piranha genera, *Serrasalmus* and *Pristobrycon*, are also the most problematic taxonomically and diagnostically. No single morphological feature has been found that completely diagnoses either, although combinations of characters have been presented to distinguish members of one piranha genus from the other and from related species (Machado-Allison 1985; Machado-Allison & Fink 1996). Some of these difficulties are exemplified by two *Serrasalmus* species, *Serrasalmus manueli* (Fernández-Yepez & Ramírez 1967) and *Serrasalmus gouldingi* (Fink & Machado-Allison 1992), found in southern Venezuela. These two large piranhas are found in Venezuela and Brazil and commonly co-occur in the Casiquiare River drainage (Fig. 4). Depending on age and environment, individual *S. gouldingi* and *S. manueli* specimens can be difficult to distinguish (Fig. 5). Most meristic and morphological characters provided to separate *S. gouldingi* from *S. manueli* show extensive overlap (Fink & Machado-Allison 1992; Machado-Allison & Fink 1996). Although intermediate-sized individuals of the two species are generally distinct in appearance, juveniles and large adults of *S. gouldingi* and *S. manueli* resemble each other closely, especially darkly-pigmented

adults (over about 250 mm TL) found in tannin-stained, blackwater habitats. Identification is occasionally, but not always, resolved when pigmentation patterns are revealed following preservation.

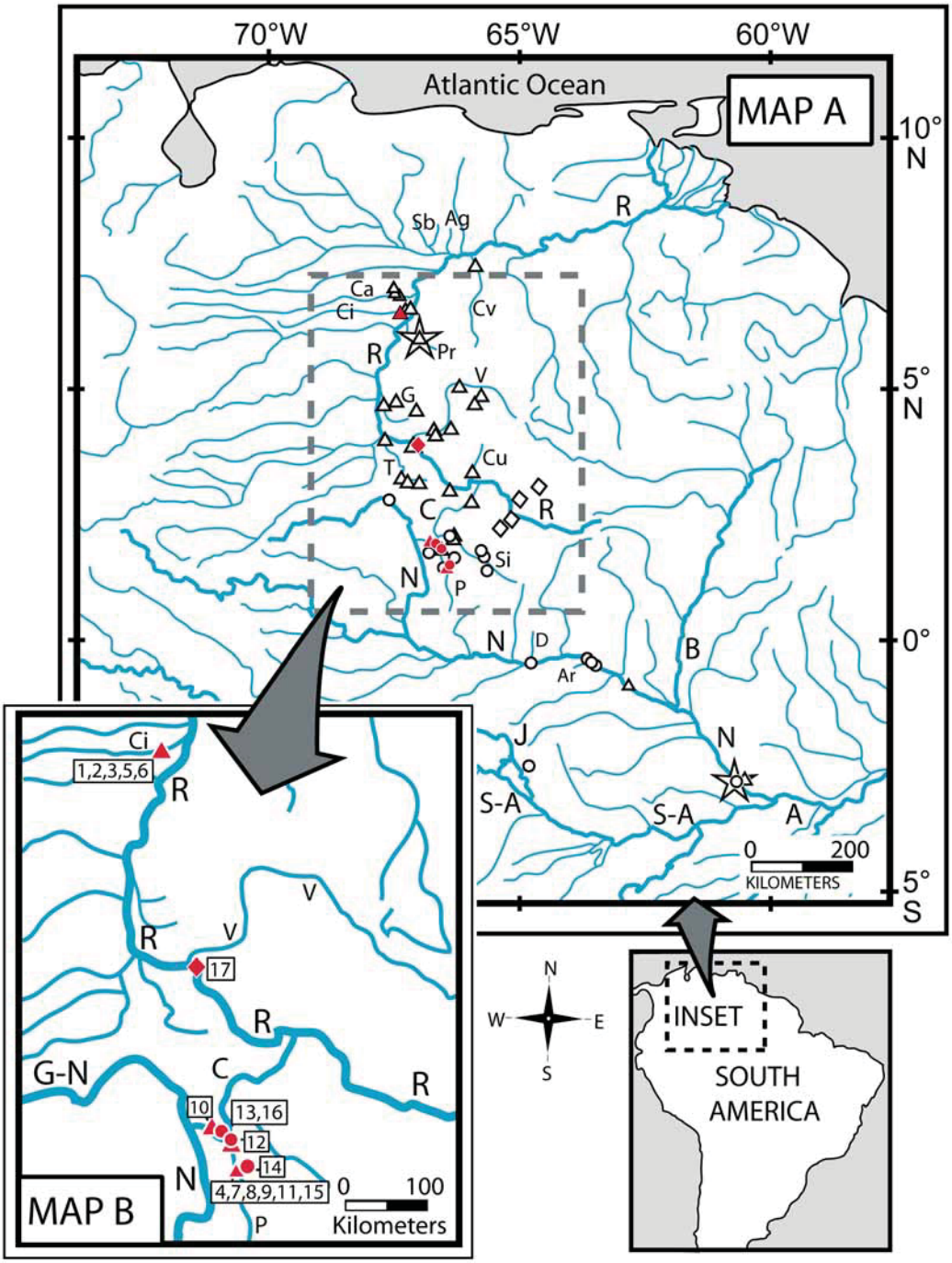

FIGURE 4. Map of northern South America showing collection sites of *Serrasalmus manueli* (triangles), *S. gouldingi* (circles), and *Serrasalmus* sp. "A" (diamond). Symbols may represent more than one collecting locality. Solid red symbols represent capture sites for material used in present genetic study (Maps A and B); numbers pertain to individual specimens, *S. manueli* (1-10), *S. gouldingi* (11-16), and *Serrasalmus* sp. "A" (17) (see Table 1). Hollow symbols on Map A are based on museum records and published information (specimen identities and capture localities were not verified for all records). Stars represent type localities for *S. manueli* (Pr, Rio Parguaza) and *S. gouldingi* (lower Rio Negro). Principal rivers: A, Amazon; B, Branco; C, Casiquiare; G-N, Guainia-Negro; J, Japurá; N, Negro; R, Orinoco; and S-A, Solimões. Other rivers: Ar, Ararirá; Ca, Capanaparo; Ci, Cinaruco; Cu, Cunucunuma; Cv, Cuchiverio; D, Daraá; G, Guaypo-Sipapo; P, Pasimoni; Pr, Parguaza; Sb, San Bartolo (Guariquito system); Si, Siapa; T, Atabapo-Atacavi; and V, Ventuari.

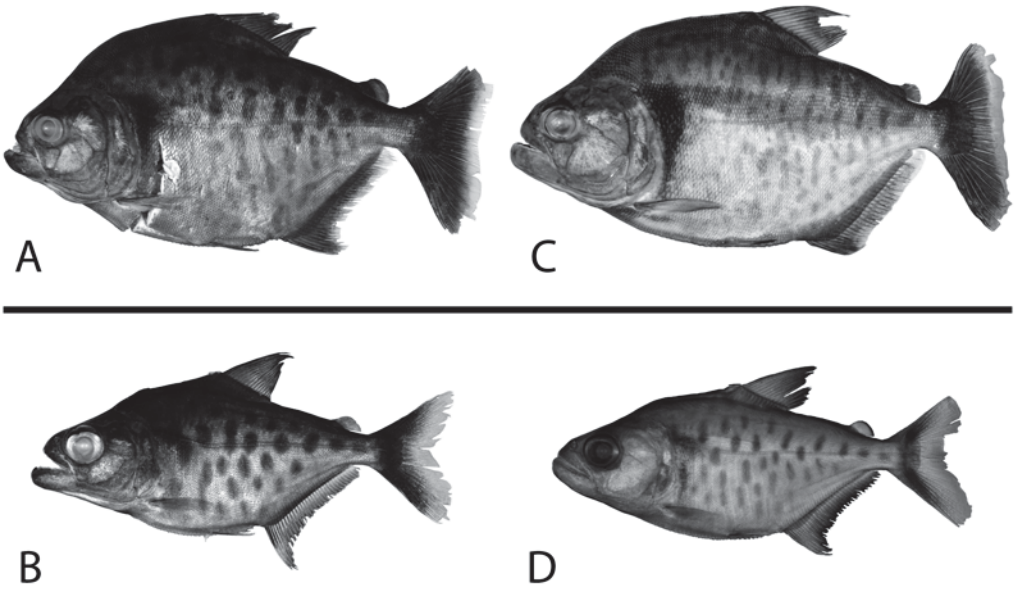

FIGURE 5. Adult and juvenile specimens of *Serrasalmus gouldingi* (A and B) and *S. manueli* (C and D) from southern Venezuela. Adult specimens (upper frame) are 195 and 240 mm SL; juvenile specimens (lower frame) are both 65 mm SL. Museum catalogue numbers for A-D: UF 148231, UF 120211, UF 121513, and UF 81180.

In addition to *S. manueli* and *S. gouldingi*, there is a *Serrasalmus* species inhabiting the upper Orinoco that we have been unable to identify. Nico (1991) referred to this piranha as *Serrasalmus* cf. *eigenmanni*, indicating its uncertain taxonomic status. We are unaware of any confirmed records of *S. gouldingi* in the upper Orinoco, but have speculated that Nico's *S.* cf. *eigenmanni*, referred to in this paper as *Serrasalmus* sp. "A", might be the whitewater form of *S. gouldingi*. Juveniles of species "A" also are similar in appearance to young *S. manueli*. (Appendix 1, Plate 3)

Material and methods

Specimen collection and vouchers. Original material, the source of tissue samples used in this study, consisted of 33 serrasalmid specimens. Of these, 31 (representing all four piranha genera and seven piranha species) were collected in the wild from the middle and upper Orinoco and upper Negro river systems during the years 1991–1992 and 1999 (Table 1; Fig. 4). Many of the source localities were in remote areas rarely or never sampled previously. Juvenile and adult specimens ranging from 28 to 275 mm SL were collected by seine, cast net, gill net, or angling. Capture localities for *S. gouldingi, S. manueli,* and *Serrasalmus* sp. "A" used in genetic analyses are shown in Figure 4. In addition, *Piaractus* tissues were obtained, one from a Venezuelan market fish and another from an introduced fish captured in a California lake. Piranha tissues (liver, muscle, caudal fin, or entire fish) for genetic analysis were either frozen, preserved in 95% ethanol, or preserved in SED (salt [NaCl], EDTA [ethylene-diamine-tetra-acetic acid], and DMSO [dimethyl sulfoxide]) buffer. Museum vouchers have been reposited for 31 of the 33 specimens (Table 1). In general, specimens were fixed in 10% formalin and later transferred to 70% ethyl alcohol. Most specimens from which tissues were removed were photographed. Depending on the specimen, images in our possession include photographs of live fish taken in the field shortly after capture or held in aquaria, preserved specimens, or combinations thereof (specimen photographs appear in Appendix 1). Radiographs of selected *Serrasalmus* and *Pristobrycon* specimens were created to examine osteological characters and verify the presence or absence of pre-anal spines.

TABLE 1. Collection localities and voucher information for original specimens used as tissue sources in this study. Refer to Appendix 1 for photographs of most specimens listed.

Specimen Identification[a]	Capture Locality Information[b]	Museum No. & size, SL [Specimen Code][c]
1) *Serrasalmus manueli*	Orinoco–Cinaruco. Field # LN99–38. Venezuela, Apure: Río Cinaruco, vicinity of Laguna Larga, 06°33'03"N, 67°24'32"W, collected by LGN, HLJ, AA, and JA, 20 Jan 1999.	UF 120067, 252 mm [LN99–38–C]
2) *Serrasalmus manueli*	Orinoco–Cinaruco. Field # LN99–38 (see above)	UF 120067, 185 mm [LN99–38–F]
3) *Serrasalmus manueli*	Orinoco–Cinaruco. Field # LN99–38 (see above)	no voucher [LN 99–38–G]
4) *Serrasalmus manueli*	Amazon–Negro –Casiquiare. Field # HLJ99–1. Venezuela, Amazonas: lagoon of Río Yatua, Río Pasimoni drainage, 01°29'00"N, 66°28'32"W, collected by HLJ, KOW *et al.*, 10 Jan 1999.	UF 136342, 265 mm [HLJ99–1–A]
5) *Serrasalmus manueli*	Orinoco–Cinaruco. Field # LN99–38 (see above)	UF 120067, 215 mm [LN99–38–D]
6) *Serrasalmus manueli*	Orinoco–Cinaruco. Field # LN99–38 (see above)	UF 120067, 208 mm [LN99–38–E]
7) *Serrasalmus manueli*	Amazon–Negro–Casiquiare. Field # KOW99–7. Venezuela, Amazonas: Río Yatua about 5 km above confluence with ríos Pasimoni and Baria, 01°29'12"N, 66°29'25"W, collected by KOW, FP, TT, and JC, 9 Jan 1999.	UF 121513, 240 mm [KOW99–7–A]
8) *Serrasalmus manueli*	Amazon–Negro–Casiquiare. Field # KOW99–7 (see above)	NLU 78895, 269 mm [KOW99–7–E]
9) *Serrasalmus manueli*	Amazon–Negro–Casiquiare. Field # KOW99–7 (see above)	UF 121513, 205 mm [KOW99–7–C]
10) *Serrasalmus manueli*	Amazon–Negro–Casiquiare. Field # LN99–05. Venezuela, Amazonas: Río Casiquiare at Raudal Yacmin, 02°00'47"N, 66°48'23"W, collected by LGN, HLJ, KOW, ABD, and HLF, 7 Jan 1999.	UF 120063, 88 mm [LN99–05–A]
11) *Serrasalmus gouldingi*	Amazon–Negro–Casiquiare. Field # KOW99–7 (see above)	UF 148231, 195 mm [KOW99-7–D]
12) *Serrasalmus gouldingi*	Amazon–Negro–Casiquiare. Field # LN99–08. Venezuela, Amazonas: lower Río Pasimoni at Laguna Arapacoa, 01°50'49"N, 66°35'07"W, collected by LGN, KOW, HLJ, ABD, HLF, FP, JC, and TT, 7 Jan 1999.	MCNG 41975, 168 mm [LN99–08–H]
13) *Serrasalmus gouldingi*	Amazon–Negro–Casiquiare. Field # LN99–31. Venezuela, Amazonas: Caño Cachiapo, left bank affluent of Río Casiquiare, 01°56'08"N, 66°41'48"W, collected by LGN, HLJ, KOW, et al., 16 Jan 1999.	UF 120211, 96 mm [LN99–31–B]
14) *Serrasalmus gouldingi*	Amazon–Negro–Casiquiare. Field # LN99–24. Venezuela, Amazonas: Río Yatua at Piedra Catipán, Río Pasimoni drainage. 01°30'58"N, 66°24'54"W, collected by LGN, HLJ, FP, KOW, and HLF, 13 Jan 1999.	MCNG 48008, 162 mm [LN99–24B]
15) *Serrasalmus gouldingi*	Amazon–Negro–Casiquiare. Field # HLJ99–1 (see above).	UF 121512, 225 mm [HLJ99-1–B]
16) *Serrasalmus gouldingi*	Amazon–Negro–Casiquiare. Field # LN99–31 (see above)	UF 120211, 65 mm [LN99–31–A]

17) *Serrasalmus* sp. "A"	Orinoco–upper Orinoco. Field #LN91-50. Venezuela, Amazonas: upper Río Orinoco at Isla El Tigre, approx. 03°55'N, 67°00'W, collected by LGN and FM, 20 Feb 1991.	UF 162488, 142 mm [LN91-50].
18) *Serrasalmus medinai*	Orinoco–Apure. Field # LN99–39. Venezuela, Apure: Caño Caicara, at bridge between Mantecal and Bruzual, 07°33'31"N, 69°15'31"W, collected by LGN, HLJ, AA, and JA, 21 Jan 1999.	UF 120212, 76 mm [LN99–39–A]
19) *Serrasalmus medinai*	Orinoco–Apure. Field # LN99–39 (see above).	UF 120212, 56 mm [LN99–39–H]
20) *Serrasalmus medinai*	Orinoco–Apure. Field # LN92–13A. Venezuela, Apure: Caño Caicara west of Mantecal, approx. 07°33'30"N, 69°20'W, collected by KOW and ABD, 23 Jan 1992.	UF 147757, 95 mm [LN92–13A–Smd]
21) *Serrasalmus irritans*	Orinoco–Apure. Field # LN99–39 (see above).	UF 120070, 51 mm [LN99–39–C]
22) *Serrasalmus irritans*	Orinoco–Apure. Field # LN99–39 (see above)	UF 120070, 49 mm [LN99–39–D]
23) *Serrasalmus irritans*	Orinoco–Apure. Field # LN99–39 (see above)	UF 120070, 55 mm [LN99–39–E]
24) *Serrasalmus irritans*	Orinoco–Apure. Field # LN99–39 (see above)	UF 120070, 80 mm [LN99–39–F]
25) *Pygocentrus cariba*	Orinoco–Apure. Field # LN99–39 (see above)	UF 120069, 95 mm [LN99–39–B]
26)*Pygopristis denticulatus*	Orinoco–Apure. Field # LN92–12. Venezuela, Apure: lagoon of Río Cinaruco, 6° 33'06"N, 67°30'43"W, collected by LGN, LMP, PC, and JL , 23 Jan 1992.	UF 147756, ~75 mm [LN92–12–Pd–01]
27) *Pygopristis denticulatus*	Orinoco–Apure. Field # LN92–12 (see above).	UF 147756, 122 mm [LN92–12–Pd–03]
28) *Pristobrycon striolatus*	Orinoco–Apure. Field # LN99–39 (see above).	UF 120068, 46 mm [LN99–39–G]
29) *Pristobrycon striolatus*	Amazon–Negro–Casiquiare. Field # LN99–12. Venezuela, Amazonas: Río Pasimoni at Laguna de Candela, 01°31'25"N, 66°33'31"W, collected by LGN, KOW, HLJ, ABD, HLF, FP, JC, and TT, 7 Jan 1999.	UF 120064, 47 mm [LN99–12–A]
30) *Pristobrycon striolatus*	Amazon–Negro–Casiquiare. Field # LN99–12 (see above)	UF 120064, 46 mm [LN99–12–B]
31) *Pristobrycon striolatus*	Amazon–Negro–Casiquiare. Field # LN99–19. Venezuela, Amazonas: Caño Cuca, left affluent of Río Pasimoni, 01°33'56"N, 66°35'18"W, collected by LGN, KOW, HLJ, ABD, HLF, FP, JC, and TT, 7 Jan 1999.	UF 120065, 24 mm [LN99–19–A]
32) *Piaractus brachypomus*	Orinoco–Apure. Field # LN93–01. Venezuela, Portuguesa: Guanare fish market, approx. 09° 05'N, 69°45'W, collected by LGN, 7 Dec 1992.	no voucher, >400 mm [LN93–1–Pb]
33) *Piaractus* cf. *brachypomus*	USA, California (introduction): San Francisco Bay basin, Sandy Wool Lake, Santa Clara County, approx. 37° 27'N, 121°52'W, collected by angler, 6 Aug 2002.	CAS 217648, 245 mm

[a]Specimen identification includes scientific name and codes used in Figures and codes associated with individual preserved vouchered specimens.

[b]Capture locality information includes river drainage, original field number, country, state, and water body, coordinates, names of collectors, and date of collection. Names of collectors are as follows: AA = Albrey Arrington, ABD = Aniello Barbarino-Duque, FP = Frank Pezold, FM = Fabian Morillo, HLF = Hernan López-Fernández, HLJ = Howard Jelks, JA

= J. Arrington, JC = James Cotner, JL = John Lyons, KOW = Kirk Winemiller, LGN = Leo Nico, LMP = Larry Page, PC = Pat Ceas, and TT = Thomas Turner.

[c]Institutional abbreviations for voucher specimens are as follows: UF = Florida Museum of Natural History (Gainesville), MCNG = Museo de Ciencias Naturales (Guanare, Venezuela), NLU = Museum of Natural History, Louisiana University at Monroe, CAS = California Academy of Sciences. Size is standard length (SL). Specimen code (field number plus additional information) represents unique alpha-numeric assigned to individual fish, especially important in distinguishing fish that are part of museum lots containing more than one specimen.

DNA extraction and PCR amplification. DNA was isolated from approximately 50 mg of tissue using standard phenol/chloroform extraction methods (Saghai-Maroof *et al*. 1984). The polymerase chain reaction (PCR) was used to amplify three portions of the mitochondrial genome: approximately 390 base pairs (bp) of the 12S rRNA, 580 bp of the 16S rRNA, and over 1200 bp of the control region and adjacent tRNAs. The 12S and 16S rRNA genes were sequenced for 33 serrasalmids in this study, including two from the genus *Piaractus*, which were used as outgroups, following Machado-Allison (1982). These sequences are deposited in GenBank under the accession numbers EF543653-EF543685 and EF543686-EF543718. In addition, complete control region sequences were generated for 25 of the above serrasalmids within our ingroup, and are deposited in GenBank under the accession numbers EF543719-EF543743. Finally, 52 serrasalmid sequences from GenBank were added for phylogenetic analyses (Appendix 2). With the exception of assigning *serrulatus* to the genus *Serrasalmus* rather than *Pristobrycon* (following Jégu 2003), we use the scientific names as they appear in GenBank (including those designating individual specimens not identified to species, e.g., "*Serrasalmus* sp. 218"). We recognize the possibility that GenBank taxonomy may be imperfect, but in the absence of voucher information, it is not possible to verify identifications and we felt it was more informative to include these sequences in spite of possible inaccuracies.

The primers used for amplification were 12SAL and 12SBH for the 12S rRNA, and 16SARL and 16SBRH for the 16S rRNA (Kocher *et al*. 1989; Palumbi 1996). The 12S BH primer is slightly modified from the original (5'- GAGAGCGACGGGCGATGTGT- 3'). Control region primers were F-TTF and F-12R (Sivasundar *et al*. 2001). Amplification of the control region included a portion of tRNA Threonine, the complete tRNA Proline, the entire control region, and a portion of tRNA Phenylalanine. Reactions were performed in a MJ Research PTC-200 thermal cycler in 50ul volumes with 1.5 mM $MgCl_2$, each dNTP at 200 micromolar, 10–100 nanograms of genomic DNA, and 1x Promega buffer B, and 1 U of Taq polymerase (Promega, Madison, WI) with a hot start and annealing temperature ranging from 49°C to 56°C. PCR products were purified with a GeneClean III Kit (Bio 101, Carlsbad, CA) or QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA). PCR products were cycle-sequenced with Big Dye version 3.1 chemistry following the manufacturer's protocol (PE-ABI) using the amplification primers for the ribosomal genes and both amplification and internal sequencing primers for the control region (Fig. 6). Sequencing reactions were analyzed on an ABI 377 automated DNA sequencer or an ABI Prism 3100 Genetic Analyzer. All samples were sequenced on both strands.

Alignment. Sequences were aligned using CLUSTAL X (Thompson *et al*. 1997) with an initial gap opening cost of 10, a gap extension cost of 2.5, and a transition weight of 0.50. Insertions deletions (indels) were uncommon in the ribosomal gene alignments. For example, an alignment of the 55 individuals for which we had both12S and 16S sequences, using the parameters listed above, generated an alignment of 988 positions. This alignment contained only 17 indel regions, only 3 of which had a length greater than 1. Indels were more common in the control region sequences, with one region in particular exhibiting great length variation among individuals that affected the overall alignment. When we analyzed this region with the program Tandem Repeats Finder 3.21, designed to find variable number tandem repeats (VNTRs, Benson 1999) it became apparent that there were between 0 and 30 copies of a complex tandem repeat in this region (Table 2). In subsequent alignments we deleted all but one copy of the repeat per individual, thereby dramatically improving

the overall alignment, although length variation remained in the region immediately surrounding the repeats. We tested the sensitivity of results by repeating all phylogenetic analyses both with and without this smaller variable region. In addition we carried out profile alignments of the control region sequences, adding taxa based on clades robustly supported in the 12S and 16S trees. No major differences were present in the various analyses. The alignment without both the tandem repeats and the associated adjacent variable length region was used for final control region analyses.

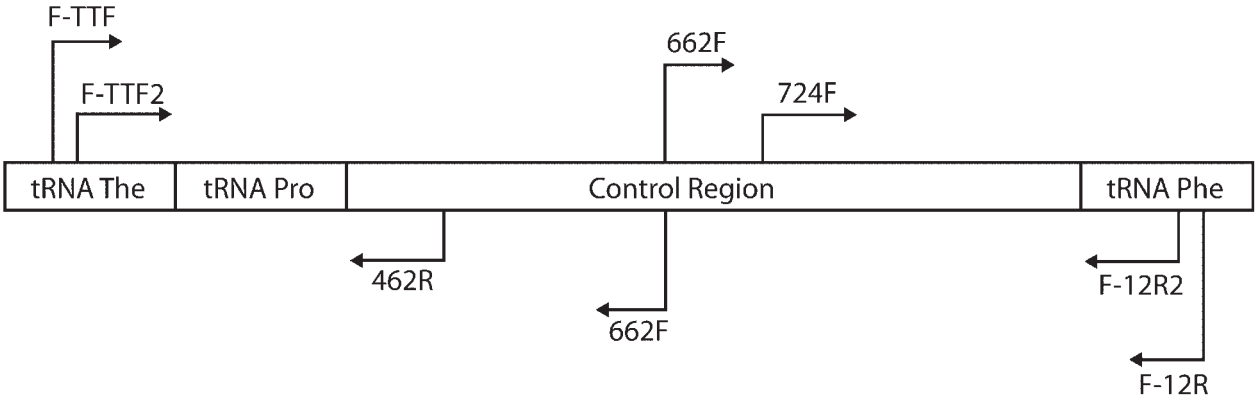

FIGURE 6. Primers used for amplifying and sequencing the control region and adjacent tRNAs. Internal primers 5'-3': 662F – ACCATGCCAAGGCGTTCTTT, 662R – AAAGAACGCCTTGGCATGGT, 724F – ACATTTGGTCACTTTCG-GAGA, 462R – CGGTTGGTGGTCTCTTACTACA, F-TTF2 – CGCCACCAGAAAAGAGAGAT, and F-12R2 - GCCCGTGGAACTTTCTAGG

Phylogenetic analyses. We carried out phylogenetic analyses of the combined 12S and 16S sequences, the control region sequences, and all three regions combined. Our phylogenetic analyses included the taxa sequenced in this study and serrasalmid sequences available in GenBank. For the multi-gene analyses we used all GenBank entries where we were able to determine that the 12S, 16S, and control region sequences were obtained from the same individual. The combined 12S and 16S data set comprised 55 individuals, the control region 63 individuals, and the combined 12S, 16S, and control region 35 individuals. Maximum parsimony analyses were carried out using PAUP* 4.0b10 (Swofford 2002) with equal weighting, uninformative characters excluded, and gaps treated as missing data. Heuristic searches with 100 random sequence additions (RSAs) and TBR branch swapping were performed followed by bootstrap analyses of 300 replicates using the heuristic option with 10 RSAs. Hierarchical likelihood ratio tests implemented in Modeltest 3.06 (Posada & Crandall 1998) were used to determine the most appropriate model of sequence evolution for each sequenced region. Results indicated that the 12S and 16S sequences and the tRNA sequences could be combined in one partition under the TrN+I+G model, while the best fit for the control region sequences was HKY+G. Bayesian analyses were performed using MRBAYES 3.0b4 (Ronquist & Huelsenbeck 2003) employing 4 Markov chains for 1 to 2 million generations and the partition-specific models described. Trees were sampled every 100th generation, and the first 10% of sampled trees were discarded as burn-in. Each run was carried out at least 4 times with different random starting trees to ensure the chains had converged to the posterior probability distribution. Graphs of generation versus log probability were examined visually for a stationary distribution. *Piaractus* was the outgroup for the separate ribosomal gene and control region analyses, while *Metynnis* was the outgroup for the combined analysis.

Zero-length branches. We used a likelihood ratio test (Goldman & Whelan 2000; Slowinski 2001) to test the null hypothesis that the branch lengths on the tree with the highest posterior probability were significantly different from zero. We used a power analysis (Walsh *et al*. 1999; Braun & Kimball 2001; Walsh & Friesen 2001) to test whether enough data had been collected to potentially resolve persistent polytomies.

TABLE 2. Variable Number Tandem Repeat (VNTR) from the control region. Taxon name is followed by the number of times repeat was present. Position numbers (1-37) and consensus sequence are presented in the first two rows."Sequences are the strict consensus of intra-individual variation in repeats. Positions that match the consensus are indicated by " . ", while gaps are represented by " – " and asterisks (*) denote indels present among repeats within an individual. VNTR variation within individuals is indicated by IUPAC (International Union of Pure and Applied Chemistry) degeneracy codes. Number preceding selected taxa corresponds to material listed in Table 1 and Appendix 1.

Taxon	**#VNTRs**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**
CONSENSUS		T	A	T	A	G	T	A	C	A	T	A	A	T	-	G	G	T	T	-	-	T	A	G	T	-	A	C	A	T	A	T	T	A	T	G	T	A
*26)*Pygopristis denticulatus*	6.4	.	.	.	M	K	.	.	.	.	.	.	.	.	-	.	S	.	Y	-	-	.	.	.	.	-	.	.	.	.	.	W	.	.	.	.	Y	.
Pygopristis denticulatus gb	5.8	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	S	.	Y	-	-	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.
Catoprion mento gb	18.7	.	.	.	.	.	.	.	.	.	.	R	.	.	-	.	.	.	.	-	-	.	.	.	.	-	.	.	.	.	-	-	-	.	.	.	.	.
28)*Pristobrycon striolatus*	5.9	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	-	-	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.
29)*Pristobrycon striolatus*	6.9	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	-	-	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.
30)*Pristobrycon striolatus*	8.9	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	-	-	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.
31)*Pristobrycon striolatus*	5.8	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	A	-	-	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.
Pristobrycon striolatus 225 gb	5.9	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	-	-	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.
**Metynnis* sp p.31 gb	3.5	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	C	-	.	.	R	.	-	.	.	.	.	W	A	.	.	.	.	.	.
Metynnis sp p.32 gb	4.2	.	.	.	.	.	.	.	.	.	.	R	.	.	-	.	A	.	C	T	-	A	.	A	.	-	.	.	.	.	T	A	.	.	.	.	.	.
**Metynnis hypsauchen* gb	2.9	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	C	C	.	.	A	.	-	.	.	.	.	.	A	.	.	.	.	.	.
**Acnodon normani* gb	29.6	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	C	C	-	.	.	A	.	-	.	.	.	.	T	A	.	.	.	.	.	.
**Ossubus xinguense* gb	2.2	W	.	Y	M	K	.	.	.	.	.	.	M	.	-	.	.	.	.	T	-	W	.	A	.	-	G	.	.	.	W	A	W	.	.	S	.	.
**Tometes* sp.246 gb	2.3	W	.	.	M	K	.	.	.	.	.	.	.	.	-	.	.	.	.	T	-	W	.	A	.	-	R	.	.	.	.	A	.	.	.	.	.	.
**Mylesinus paucisquamatus* gb	2.0	W	.	.	M	K	.	.	.	.	.	.	.	.	-	.	.	.	.	T	-	W	.	A	.	-	R	.	.	.	.	A	.	.	.	.	.	.
Myleus pacu 69 gb	1.0	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	T	-	A	.	A	.	-	G	.	.	.	.	A	.	.	.	.	.	.
Myleus pacu 70 gb	1.0	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	T	-	A	.	A	.	-	G	.	.	.	.	A	.	.	.	.	.	.
**Myleus* sp. p49 gb	2.5	W	.	Y	M	K	.	.	.	.	.	.	.	.	-	.	.	.	.	T	-	W	.	A	.	-	.	.	.	.	W	A	.	.	.	.	.	.
**Myleus* sp. p51 gb	2.1	W	.	W	M	K	.	.	.	.	.	.	M	.	A	.	.	.	Y	T	-	W	.	A	.	A	R	.	.	.	W	A	.	.	.	.	.	.
**Myleus* tiete gb	2.2	W	.	.	M	K	.	.	.	.	.	.	R	.	-	.	.	.	.	T	-	W	.	A	.	-	R	.	.	.	W	A	.	.	.	.	.	.
**Myleus* asterias gb	2.0	W	.	.	M	K	.	.	.	.	.	.	.	.	-	.	.	.	.	T	-	A	.	A	.	-	.	.	.	.	.	A	.	.	.	.	.	.
**Myleus* rubripinnis gb	2.0	W	.	.	M	K	.	.	.	.	.	.	.	.	-	.	.	.	.	T	-	A	.	A	.	-	.	.	.	.	W	A	.	.	.	.	.	.
**Myleus* ternetzi gb	2.2	W	.	Y	M	K	.	.	.	.	.	.	.	.	-	.	.	.	Y	T	-	W	.	A	.	-	R	.	.	.	M	A	.	.	.	.	.	.
Colossoma macropomum gb	1.5	.	.	.	.	.	.	.	.	.	.	T	.	.	-	.	.	.	A	-	-	.	.	.	.	-	.	.	.	.	T	A	.	.	.	.	Y	.
Mylossoma duriventri gb	1.5	.	.	.	.	.	.	.	.	.	.	T	.	.	-	.	.	.	A	-	-	.	.	R	.	-	.	.	.	.	W	A	.	.	.	.	Y	.
Mylossoma paraguayensis gb	1.5	.	.	.	.	.	.	.	.	.	.	T	.	.	-	.	.	.	A	-	-	.	.	R	.	-	.	.	.	.	W	A	.	.	.	.	Y	.
**Piaractus mesopotamicus* gb	1.7	.	.	.	.	K	.	.	.	.	.	.	.	.	-	.	A	.	C	C	C	-	.	.	.	-	R	.	.	.	T	A	.	.	.	R	.	.
Piaractus brachipomus 58 gb	1.5	.	.	.	.	.	.	.	.	.	.	T	.	.	-	.	.	.	A	-	-	.	.	.	.	-	.	.	.	.	W	W	.	.	.	.	.	.
**Piaractus brachipomus* 60 gb	2.0	.	.	.	M	K	.	.	.	.	.	T	.	.	-	.	.	.	A	-	-	.	.	.	.	-	.	.	.	.	W	W	.	.	.	.	.	.

Results

Sequence characteristics. The amplified portions of the 12S and 16S genes were approximately 390 and 580 bp in length. The mean base frequencies were A= 0.304, C= 0.256, G= 0.228, and T= 0.212. Base frequencies did not vary significantly among taxa, based on the chi-square test implemented in PAUP*. Of 988 aligned positions, 149 were variable and 113 were parsimony informative. Uncorrected pairwise differences for this data set were between 0 and 7 percent.

The amplified region that included the control region and tRNAs ranged in size from 1216 to 1471 bp in the taxa we sequenced. As previously discussed, the length variation was due mainly to a single region of variable number tandem repeats (VNTRs), which are common in vertebrate control region sequences (Ludwig *et al*. 2000; Lunt *et al*. 1998; Ravago *et al*. 2002; Ray & Densmore 2002). The control region and adjacent tRNA alignment was 1253 positions, of which 389 were variable, and 292 were parsimony informative. When GenBank sequences were added, length varied between 1,069 and 1,887 bases with repeats included, and from 969 to 1230 bases with all but one repeat removed. The mean base frequencies were A= 0.308, C= 0.238, G= 0.166, and T= 0.288, and base frequencies did not vary significantly among taxa based on the chi-square test.

Phylogenetic analyses. The species *Serrasalmus manueli* and *S. gouldingi* were both monophyletic in analyses based on the combined 12S, 16S, and control region sequences we generated in this study. Parsimony and Bayesian analysis of our combined sequences recovered monophyletic *S. manueli* and *S. gouldingi* clades with 100% bootstrap proportion (BP) and posterior probability (PP). Similarly, analysis of our 12S and 16S sequences combined with those available from GenBank recovered a well-supported *S. manueli* clade and an *S. gouldingi* clade with 68% bootstrap value and 100% posterior probability (Fig. 7A). Our control region sequences combined with available GenBank sequences recovered a monophyletic *S. manueli*, but failed to support the monophyly of *S. gouldingi* (Fig. 7B). When we combined our 12S, 16S, and control region sequences with those serrasalmid individuals in GenBank for which all three genes were available, we once again recovered a monophyletic *S. manueli*, but *S. gouldingi* appeared paraphyletic with respect to *Serrasalmus* sp. "A", *Pristobrycon* specimen 224, and *Serrasalmus spilopleura* (Fig. 8).

There was no support for the monophyly of *Serrasalmus* in our analyses. Representatives of the genus *Serrasalmus* typically formed unresolved polytomies with *Pygocentrus cariba*, *Pygocentrus nattereri*, and *Pristobrycon* sp. (Fig. 7). In some cases, there was support for the paraphyly of *Serrasalmus* with respect to *Pygocentrus* and *Pristobrycon* sp. 224 (Fig. 8), "*Pristobrycon" serrulatus*, or *Pristobrycon* sp. (Fig. 7B). *Pristobrycon* was polyphyletic in all analyses that included more than one species of *Pristobrycon*, although, as previously noted, *Pristobrycon serrulatus* is treated as *Serrasalmus serrulatus* by some recent authors. In the rRNA data set there was moderate parsimony support and strong support in the Bayesian analysis for a clade of *Serrasalmus compressus, S. rhombeus, S. humeralis*, *Serrasalmus* sp., *Serrasalmus* sp. 218, and *Serrasalmus* sp. 219 (Fig. 7A). In fact, the three specimens in this clade identified as *Serrasalmus* sp. are identical over the 988 positions of the ribosomal RNA alignment with *S. compressus*, suggesting these may be conspecific.

Finally, several suprageneric clades were also supported within the piranha. A clade of all *Serrasalmus*, plus *Pygocentrus* and part of *Pristobrycon* (*Pristobrycon* sp.) was robustly supported in all analyses. All of the taxa in this clade also lack the control region repeat. In addition, a clade composed of *Catoprion* and *Pygopristis* was well supported across analyses, with *Pristobrycon striolatus* as sister to this clade. The monophyly of the four piranha genera, with *Catoprion*, was also found in all analyses.

Discussion

Mitochondrial data presented here support some previous hypotheses of evolutionary relationships and pro-

vide evidence of unsuspected relationships among serrasalmids. These data also bear on questions about the timing of divergence events within this clade. Results for specific clades are as follows:

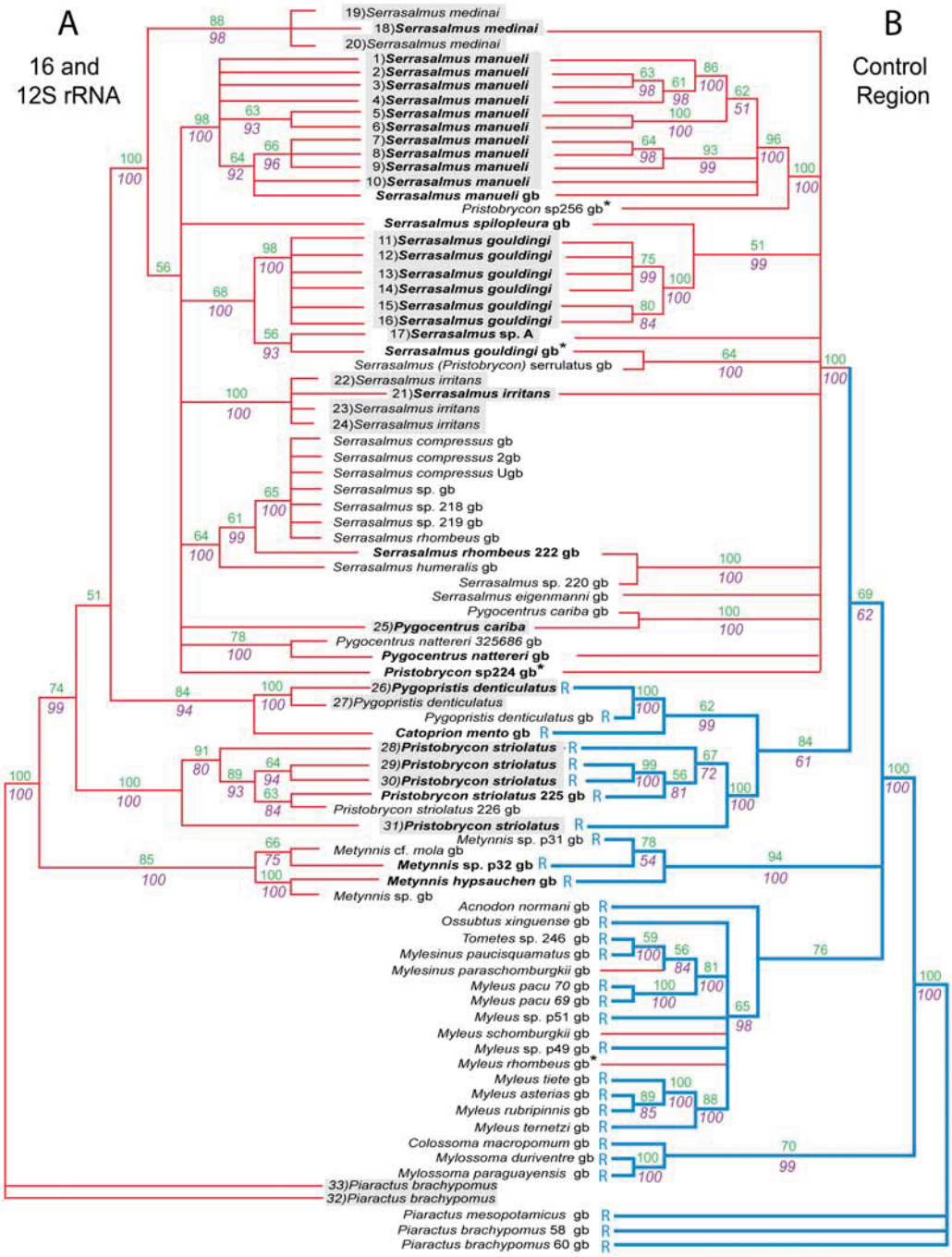

FIGURE 7. Phylogenetic trees of serrasalmids inferred from ribosomal (A) and control region (B) data sets, both including original material and GenBank sequences. Parsimony bootstrap percentages are shown above branch nodes, while proportions (>50%) of trees possessing a given clade in Bayesian posterior distributions are shown below. Taxa shared by both trees are in bold font. Specimen sequences from original material appear in shadow boxes, preceding numbers (1-33) correspond to numbered specimens and information presented in Table 1 and elsewhere. GenBank sequences are followed by gb. Taxa with VNTR in control region marked by an "R" and reconstruction of presence of VNTR shown in blue. GenBank (gb) species with asterisk (*) indicate taxa of which we question the identification.

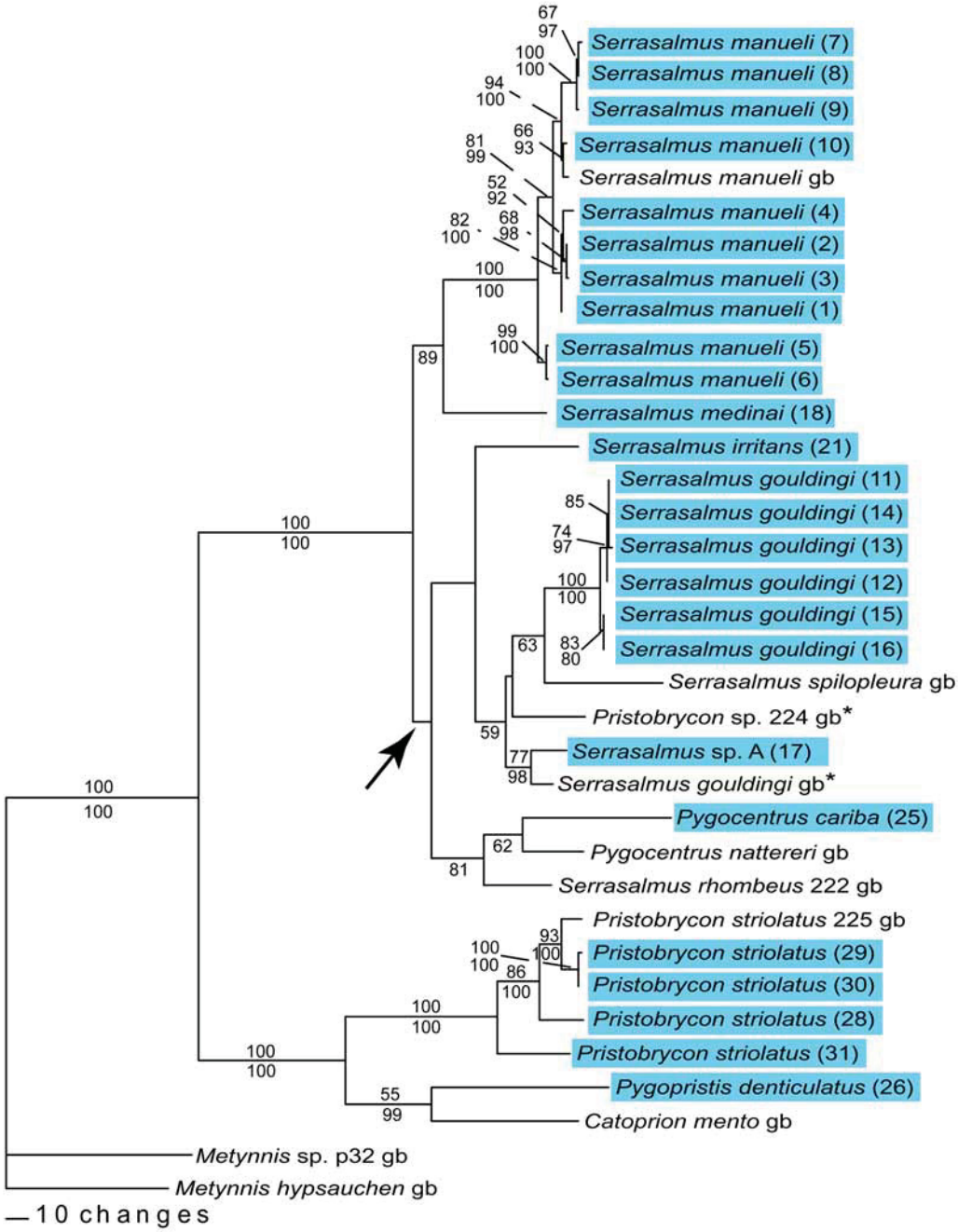

FIGURE 8. Phylogram of combined ribosomal and control region sequences. Analysis includes specimens appearing in bold font in Figure 7. Proportion of trees from posterior distribution possessing a given clade below branch, parsimony bootstrap proportions (>50%) above branch. Specimen sequences from original material appear in shadow boxes, associated number in parentheses (1–33) correspond to numbered specimens and information presented in Table 1 and elsewhere. GenBank sequences are followed by gb. Arrow marks branches with lengths that were not significantly different from zero. GenBank (gb) species with asterisk (*) indicate taxa of which we question the identification.

***Serrasalmus* and *Pristobrycon*.** Our mitochondrial data do not support the reciprocal monophyly of *Pristobrycon* and *Serrasalmus* as defined by Machado-Allison (1983, 1985), in agreement with Ortí *et al*. (1996) and others. This conclusion depends of course, on proper generic placement, which is in doubt for some species. For example, one problematic taxon is the piranha species *serrulatus*, which Ortí *et al*. (2000) treated as *Pristobrycon serrulatus*. Jégu (2003), in contrast, listed *serrulatus* under the genus *Serrasalmus*, but without explanation. Jégu and Dos Santos (1988) reported that the holotype for *S. serrulatus* (MNHN A9858, 117 mm

SL) had ectopterygoid teeth. In contrast, Goulding (1980:166) stated that *serrulatus* lacked palatine (i.e., ectopterygoid) teeth. Absence of these teeth suggests the possibility that Goulding's study animals (130-250 mm SL), all from the Madeira River drainage of the Brazilian Amazon, were a species of *Pristobrycon*. However, ectopterygoid teeth are often not replaced, so these teeth may be absent in some older *Serrasalmus* individuals (for additional discussion see subsequent section on morphological characters). Similar to the situation with *serrulatus*, the recent literature is inconsistent concerning generic placement (typically *Serrasalmus* versus *Pristobrycon*) of a number of other piranhas, for example, *aureus*, *eigenmanni,* and *spilopleura*. This uncertainty directly influences testing the monophyly of some nominal genera.

Our molecular results indicate that *Serrasalmus compressus*, *S. rhombeus*, *S. humeralis*, and one or more unidentified *Serrasalmus* species form a monophyletic subgroup (Fig. 7) within *Serrasalmus*. Results from our combined rRNA and control region sequences provided weak evidence for a clade consisting of *Serrasalmus rhombeus* and *Pygocentrus* (*P. carib*a and *P. nattereri*) (Fig. 8), but this is contradicted by anatomical evidence (see Machado-Allison 2002).

The genus "*Pristobrycon*" as currently construed, is not monophyletic. The most studied member of the genus is *P. striolatus*, a widespread species found in the Amazon, Orinoco, and Guiana drainages. Mounting evidence indicates it is distinct both genetically and morphologically from other *Pristobrycon* species (Machado-Allison 1985; Ortí *et al*. 1996, 2000; Nakayama *et al*. 2002, herein).

Other piranhas and related genera. Our combined genetic analysis suggests that *Pygopristis* is more closely related to *Catoprion* than to any other piranha genus, a result also found by Ortí *et al.* (2000). The monophyly of the four genera of the traditional "true piranhas" (see Introduction) is not supported by the mtDNA data. If the terminology is intended to apply to a monophyletic assemblage of genera it should either be expanded to include *Catoprion*, or reduced to only three genera (i.e., *Serrasalmus*, *Pygocentrus*, and *Pristobrycon* [without *P. striolatus*]) by excluding *Pygopristis*.

Piranhas of southern Venezuela. Our study focused on three piranha species found in southern Venezuela, *Serrasalmus manueli*, *S. gouldingi*, and an unknown species that we refer to as *Serrasalmus* sp. "A" (Fig. 5; Appendix 1). Difficulty in identifying live and preserved specimens at different life stages was the impetus for us to use molecular data to distinguish and verify identifications of these three taxa. The situation is complicated by the fact that many piranhas coexist in the enormous region that includes the upper Orinoco and Negro river systems.

Serrasalmus manueli. Our molecular data, based on 11 specimens (10 new and 1 GenBank), indicated that *S. manueli* is monophyletic (Figs. 7, 8). The five *S. manueli* from the Cinaruco River (Orinoco Basin) and the five *S. manueli* captured in the Casiquiare (Negro River drainage) were not reciprocally monophyletic. There does, however, appear to be a geographic structuring based on limited sampling, with the majority of the Negro River drainage *S. manueli* being part of a well-supported monophyletic clade, and the Orinoco samples forming a paraphyletic assemblage at the base of this clade. Because of their morphological similarity and our preliminary distribution information (see subsequent discussion on biogeography), we originally suspected the sister group of *S. manueli* was *S. gouldingi*. Our genetic results provide no support for that assumption, although there is some evidence that *S. medinai* is more closely related to *S. manueli* than is *S. gouldingi* (Fig. 8). At intermediate sizes, *S. manueli* is fairly distinct, largely because of the combination of body shape and pigmentation pattern. Identification of juveniles is problematic because of their similarity to young of several other piranha species. In addition, large *S. manueli* inhabiting blackwater systems are darkly pigmented, obscuring critical marks for identification. In these cases, our molecular data resolved species identification.

Serrasalmus gouldingi. Our sample of six *S. gouldingi* specimens, from the Negro-Casiquiare region, was monophyletic. In contrast, the single *S. gouldingi* GenBank specimen was more closely related to other taxa in some analyses, for example, to "*Pristobrycon" serrulatus* from GenBank for the control region (Fig.

7) and to *Serrasalmus* sp. "A" for 16S and 12S rRNA (Fig. 7) and the combined ribosomal and control region sequences (Fig. 8). These results suggest that either *S. gouldingi* is not monophyletic, or that the GenBank specimen is not *S. gouldingi*. This specimen has been identified both as *S. gouldingi*, and *S. sp.* so this identification could be considered tentative. We collected a series of small juvenile piranhas, taken in the same locality and habitat as adult *S. gouldingi*. Based on their proximity of capture and similarity in general appearance, in the field we tentatively identified the juveniles as young of the adult *S. gouldingi*. After considering the molecular data, we reexamined the preserved material and determined the juveniles were *Pristobrycon striolatus*.

***Serrasalmus* sp. "A".** This unidentified species, whose relationship to other piranhas is unclear, was represented in our analysis by a single specimen taken from the mainstem of the upper Orinoco River. As mentioned above, *Serrasalmus* sp. "A" consistently groups with the GenBank "*S. gouldingi*" in the control region analysis (Fig. 7) and in the combined analysis (Fig. 8). One possible explanation is that *Serrasalmus* sp. "A" and GenBank "*S. gouldingi*" represent a single species whose identity is yet to be satisfactorily determined. Nico (1991) used the name *Serrasalmus* cf. *eigenmanni* for specimens that we consider *Serrasalmus* sp. "A", although it is possible that his 48 specimens include other problematic taxa. Prior to our genetic analysis, we thought that *Serrasalmus* sp. "A" might simply be the "whitewater form" of *S. gouldingi*. Our combined ribosomal and control region analysis results, however, indicates it is distinct from *S. gouldingi* (Fig. 8).

Serrasalmus species "A" is morphologically similar to a group of other poorly-defined piranhas, including *Serrasalmus eigenmanni* Norman 1929, *S. humeralis* Valenciennes 1850, *S. nalseni* Fernández-Yépez 1969, *S. serrulatus* (Valenciennes 1850), among others, that have confused systematists and other piranha researchers. In general, these piranhas are small to moderate in size (< 200 mm SL), have a moderately deep body, slightly pointed snout, and the caudal fin base is heavily pigmented forming a dark crescent. The pattern of spotting on the sides is variable with spots often irregular in shape. With age, spots may merge to form larger spots, some vertically elongate. A humeral blotch, if present, is typically faint and slightly elongate vertically. The humeral blotch and side spotting often are not apparent except in preserved specimens. The body shape and pigmentation pattern of *Serrasalmus* sp. "A" varies considerably with growth. Juveniles have a pointed snout and somewhat elongate body, but in larger individuals the snout is only slightly pointed and the body much deeper relative to body length. Similar to *S. gouldingi*, large adult *Serrasalmus* sp. "A" may have black pigments extending across nearly the entire tail except for a terminal hyaline band.

We recently reexamined four *Serrasalmus* sp. "A" specimens collected by Nico (1991), including the source of our genetic tissue, and determined that all had pre-anal spines. We partially dissected one of these, a museum specimen (UF 85215, Field Number LN 91-39; 140 mm SL) collected in the Mavaca River and were unable to detect ectopterygoid teeth. As discussed previously, the absence of these teeth in adults may not be diagnostic.

A persistent polytomy in the piranha clade. Analysis of the ribosomal RNA data set, with 149 variable and 113 parsimony informative characters, resulted in a large polytomy at the base of the *Serrasalmus*, *Pygocentrus*, *Pristobrycon* (excluding *P. striolatus*) clade (Fig. 7). The retention index of this data set (0.86) did not indicate excessive character conflict as the cause of the polytomy, so we attempted to increase resolution by the addition of complete control region sequences. The control region sequences added 560 variable characters and 385 parsimony informative characters. Despite having more than quadrupled the number of variable or parsimony informative characters, the polytomy persisted (Figs. 7, 8). The persistence of this polytomy with increased sampling suggested that it might represent a molecular polytomy. By molecular polytomy we mean either simultaneous divergence of three or more genes from an ancestral gene (a hard molecular polytomy) or a case in which divergence may not have been simultaneous but in which branch lengths between divergence events are so short that too few substitutions have accumulated to resolve the order of branching (a soft molecular polytomy) (Slowinski 2001).

We used a likelihood ratio test on the tree with the highest Bayesian posterior probability (Fig. 8) to determine whether we could reject the hypothesis that the branches at the base of this clade were zero-length (Goldman & Whelan 2000; Slowinski 2001). We used the corrected chi-square for one degree of freedom at 95% from Table 2 in Goldman and Whelan (2000). We were unable to reject the hypothesis that the branches were zero-length for many terminal nodes, and more importantly for an internal node marked by an arrow in Figure 8. Tree topologies and branch length tests therefore suggest a hard or soft mitochondrial molecular polytomy at the base of the *Serrasalmus*, *Pygocentrus*, and *Pristobrycon* (excluding *P. striolatus*) clade.

There are several possible explanations for this polytomy. One simple explanation is loss of phylogenetic signal due to saturation. The robust support for clades both above and below this polytomy argues against this explanation. A second, related argument is that the substitutional dynamics of piranha mitochondrial DNA might result in an area of poor resolution in the middle of the tree even if branch lengths in the region of the polytomy were not significantly shorter than in other portions of the tree in terms of absolute time. For example, more rapidly accumulating transitional changes might lend resolution in the tips of the tree, and more slowly accumulating transversions would resolve the deeper branches of the tree, but there might be a zone in the middle of the tree where transitional changes were beginning to saturate and lose signal, while too few transversional changes had accumulated to provide adequate resolution. We used MacClade to infer the average number of unambiguous transitions and transversions in different parts of this clade. The transition-transversion ratio was 3.5 above the polytomy, 4.4 in the short branches that make up the polytomy, and 1.3 below the polytomy, consistent with this scenario. If the molecular dynamics of mitochondrial DNA in piranha are the cause of this polytomy, we would expect that other unlinked genes with differing substitutional dynamics, for example nuclear genes, would robustly support a resolved phylogeny in this portion of the tree.

One method for addressing whether a polytomy is soft is to use a power analysis to determine if, based on the substitution rate of the gene in question, there are enough data to resolve a given polytomy (Walsh *et al.* 1999; Braun & Kimball 2001; Walsh & Friesen 2001). Donaldson and Wilson (1999) analyzed control region sequences in sister snook species (*Centropomis*) separated by the emergence of the Isthmus of Panama 3.5 million years ago to derive an annual rate of 1.8×10^{-8} substitutions. Applying a p-value of 0.05 and a Poisson algorithm (equation 2, Walsh & Friesen 2001), we estimate a 95% chance of differentiating serrasalmid speciation events between 150,000 and 194,000 years ago using only 986 bp of control region sequence. This suggests that, unless speciation events have happened very recently or our substitution rate is not appropriate, our data should be sufficient to detect it. Finally, the molecular polytomy might represent a hard or soft species polytomy. If this were the case, we would expect other unlinked genes to show a polytomy, or poorly supported and conflicting resolutions in the area of the polytomy. Sampling nuclear genes from these taxa should allow us to test these competing explanations.

We estimated the time of divergence of this polytomy by applying the control region divergence rate calculated for snook (*Centropomis*), 3.6 % per million years (Donaldson & Wilson 1999), to our data. The rate was applied to uncorrected p-values for all pairwise comparisons that passed through the polytomy node (arrow in Fig. 8). The average divergence time estimated by this method was 1.8 million years. This corresponds to the beginning of the Pleistocene, a time of increasingly seasonal and more cyclic climate. These climatic cycles, which are thought to have resulted in cyclical fragmentation and coalescence of habitats, could drive simultaneous divergence resulting in true species polytomies. An important caveat to this analysis is that there is not widespread agreement on the rate of divergence of the control region in characiform fishes. The calibration chosen is independent of our analysis but other lower rates (e.g., Sivasundar *et al.* 2001) would result in a considerably older date for this polytomy. Lundberg (1997, 1998), based on a fossil tooth from the La Venta fauna assigned to either *Pygocentrus*, *Pristobrycon*, or *Serrasalmus*, and further assuming the monophyly of these genera, concluded the piranha-like serrasalmids had evolved by the late Middle Miocene, approximately 11 million years ago. Reis (1998), based on a similar analysis, suggested that these genera may have originated after the Middle Miocene. Our phylogenetic analysis indicates that *Pygocentrus*, *Pristobry-*

con, and *Serrasalmus* are not part of a monophyletic clade, and, in addition, the genus *Pristobrycon* is not monophyletic. This undermines the phylogenetic rationale for the fossil dating, and therefore the value of this tooth-type as a synapomorphy for the clade in question. The timing of origin of the piranha-like serrasalmids must be considered an open question, but may be considerably more recent than the Middle Miocene.

Corroboration from variable number tandem repeats (VNTRs) and morphology. Our sequence-based molecular analysis provides evidence for a clade formed by the genera *Serrasalmus*, *Pygocentrus*, and *Pristobrycon* (excluding *P. striolatus*) and there is robust support across all analyses for monophyly of this group (Figs. 7, 8). In addition, all of the members of this clade lack the control region VNTR. In contrast, the VNTR is present in the majority of the remaining serrasalmid genera sampled (Table 2; Fig. 7, VNTR marked with the letter R). We randomly resolved the polytomies in this tree 100 times, and reconstructed the gain or loss of the VNTR. In all reconstructions, the VNTR is lost at the base of *Serrasalmus*, *Pygocentrus*, and *Pristobrycon* (excluding *P. striolatus*) clade. This adds a unique complex molecular character further supporting the monophyly of the *Serrasalmus*, *Pygocentrus*, and *Pristobrycon* (excluding *P. striolatus*) clade. Members of this clade sampled to date also share certain morphological traits, for example a pre-anal spine and ectopterygoid teeth (see Fig. 1B).

The genera *Serrasalmus* and *Pristobrycon* include most of the currently recognized piranha species (Jégu 2003). However, morphological diagnosis of these two genera is problematic and depends heavily on combinations of characters. Some of these characters exhibit considerable variation within genera and even within particular species. This unsatisfactory situation was highlighted by Fink and Machado-Allison (1992) when they noted that some of the features used to diagnose *Serrasalmus* by Machado-Allison (1985) do not apply when a larger number of species than he had available are examined. As a result, it is difficult to find obvious relationships between particular morphological characters and the molecular data. Within the piranha clade, two main characters considered to be derived are the pre-anal spine and ectopterygoid teeth. These are worth examining in detail, although determination of the degree of correspondence to our molecular phylogenies will require further sampling of many species for both morphological and molecular data. As noted above, our results suggest a possible correspondence between a well-supported clade in our sequence based phylogeny, presence-absence of the control region VNTR and presence-absence of a pre-anal spine within piranhas and their close relatives. Piranhas without the VNTR (*Pygocentrus* + *Serrasalmus* + *Pristobrycon* [in part]) have a pre-anal spine (see Fig. 1B). Others (*Pristobrycon* [in part] + *Pygopristis* + *Catoprion*) have the VNTRs, but do not have a pre-anal spine. The only serrasalmid taxa that we sampled that lack the VNTRs and are not part of our *Serrasalmus*, *Pygocentrus*, and *Pristobrycon* (in part) clade are *Mylesinus paraschomburgkii*, *Myleus schomburgkii*, and *Myleus rhombeus* (Fig. 7), although these genera and species all presumably lack a pre-anal spine.

The pre-anal spine is a bony element at the anal fin origin. According to Fink and Machado-Allison (1992), it is not visually apparent but the structure can usually be detected by touch as a sharp process. Because alcohol preservation typically shrinks soft tissues, the pre-anal spine is often more exposed in preserved specimens, particularly in juveniles and some piranhas with highly compressed bodies (e.g., *Serrasalmus irritans*). However, the structure may still be difficult to locate in large piranha specimens and confirmation of its presence often requires radiographs (see Fink & Machado-Allison 1992). Machado-Allison (1985, 2002) considered the pre-anal spine to be a derived character. Among the "true piranhas" it is present in all members of the genera *Serrasalmus* and *Pygocentrus* but absent in *Pygopristis*. Moreover, the pre-anal spine is present in some, but not all, species of the genus *Pristobrycon* (Géry 1972; Machado-Allison 2002). It has been suggested that *P. striolatus* is the only species within the genus lacking a pre-anal spine (Ortí *et al.* 1996), but this is not the case. For example, at least three of the four *Pristobrycon* species known to occur in Venezuela do not have a pre-anal spine (i.e., *P. striolatus*, *P. maculipinnis*, and *P. careospinus*) (Fink & Machado-Allison 1992; Machado-Allison & Fink 1996). In contrast, *Pristobrycon calmoni* and reportedly

a few other members of the genus have a pre-anal spine (Jégu & Dos Santos 1988; Machado-Allison & Fink 1995). Based on the available information, the pre-anal spine is apparently absent in all of the remaining serrasalmid genera, for example, *Catoprion* and *Metynnis* (Machado-Allison et al. 1989); *Mylossoma*, *Piaractus*, and *Colossoma* (Ortí *et al*. 1996); and *Myleus* (based on our own examination of *M. torquatus* specimens).

Ectopterygoid teeth (referred to as palatine teeth in some piranha literature) are small teeth situated on the roof of the inner mouth. The presence versus absence of these teeth is often used in identification keys because the ectopterygoid teeth are present in all *Serrasalmus* and some *Pristobrycon*. In contrast, according to Fink (1993), *Pygopristis* and *Pygocentrus* of all ages lack ectopterygoid teeth (but see Machado-Allison 1985, 2002). Unfortunately the situation is complicated. In many *Serrasalmus* and *Pristobrycon* species replacement of ectopterygoid teeth is not continuous throughout ontogeny and these teeth may be absent in large individuals (Fink 1993). Loss of ectopterygoid teeth with age has been documented for most *Serrasalmus* species studied in detail: *S. altuvei*, *S. compressus*, and *S. geryi* (Jégu *et al.* 1991); *S. gouldingi* and *S. manueli* (Fink & Machado-Allison 1992); *S. altispinis* (Merckx *et al.* 2000); *S. maculatus* (Jégu & Dos Santos 2001), and *S. rhombeus* (Machado-Allison 2002), among others. The size or age at which individuals no longer have ectopterygoid teeth varies considerably among species. For example, in their original description of *S. gouldingi* Fink and Machado (1992) remarked that replacement of these teeth ceases early in life so most adults lack them. However, in some species a few of the larger adults still retain at least a few of these teeth (e.g., *S. compressus*, Jégu *et al.* 1991). Although certain *Pristobrycon* have ectopterygoid teeth, these teeth are fewer and differently shaped than those in *Serrasalmus* (Machado-Allison 2002). In *Serrasalmus* their shape is similar to that of the jaw teeth (i.e., triangular and often with cusps) whereas *Pristobrycon* ectopterygoid teeth are relatively wide, square, and blunt.

There are additional complications concerning this character among other serrasalmid taxa. Fink (1993) stated that *Pygocentrus* lacked ectopterygoid teeth at all ages, but Machado-Allison (2002:56) noted that early juveniles (>10 mm SL) do possess 6 or more minuscule, unicuspid teeth on the ectopteryogoid bone. Previously, Machado-Allison (1985:33) reported the adults of some other serrasalmid genera have edenticulate ectopterygoides and the juveniles of *Pygocentrus*, *Pygopristis*, *Mylossoma*, *Colossoma*, and *Piaractus* have small conical teeth on the ectopterygoid that temporarily form during development, a situation that Machado-Allison remarked as apparently representing a plesiomorphic character among members of the family Characidae. Machado-Allison (1985) suspected the strongly tricuspid ectopterygoid teeth found in *Pristobrycon* and *Serrasalmus* were a specialization that distinguished them from the unicuspid (primitive) condition present in other serrasalmids. Based on this information, the presence of ectopterygoid teeth, even if only temporarily in *Pygocentrus*, combined with pre-anal spines and absence of the VNTR, are indicative of all *Pygocentrus* and *Serrasalmus* and some *Pristobrycon*.

Machado-Allison (1982, 1983) considered *Pristobrycon* to be more closely related to *Serrasalmus* than any other group in the family (Fig. 1A), although Machado-Allison (1985) later concluded that an exhaustive revision of the genus was necessary to establish precise limits of the taxa. In a subsequent review, Machado-Allison (2002) addressed some anatomical characters helpful in delimiting *Pristobrycon* and *Serrasalmus* and commented that the reanalysis of some characters suggests that *Pristobrycon* should be divided into two subgroups, one that includes species with a pre-anal spine and the other without this character. Although this view is a slight modification to the cladogram of Machado-Allison (1985), the idea that existing *Pristobrycon* can simply be considered two "subgroups" within the genus clashes with the alternative cladogram of Machado-Allison *et al*. (1989) (compare Figs. 1A and 1B). The revised hypothesis of Machado-Allison *et al*. (1989) recognized the absence of the pre-anal spine as a plesiomorphic "primitive" character, and, in particular, suggested paraphyly of *Pristobrycon.* Based on our review of literature, those *Pristobrycon* species without ectopterygoid teeth reportedly also lack pre-anal spines (Jégu & Dos Santos 2001; Machado-Allison 2002). However, the published information is incomplete, highlighting the need to examine a wider range of juvenile and adult specimens and confirm whether ectopterygoid teeth are absent at all sizes (and not just in adults). If this anatomical dichotomy holds, then it lends additional support to the hypothesis that the species now gener-

ally considered to make up the genus *Pristobrycon* are actually two distinct groups that should be recognized as separate genera that are not sister taxa.

A conceivable remedy is to simply transfer any "*Pristobrycon*" species with pre-anal spines and ectopterygoid teeth into the genus *Serrasalmus*. Unfortunately when Eigenmann (1915) erected the genus *Pristobrycon*, he unwittingly created problems for later systematists by designating *P. calmoni* as the type for the genus, a species with a pre-anal spine and, at least in juveniles, ectopterygoid teeth (Jégu & Dos Santos 1988; but see Machado-Allison 2002). Given the above, there appears justification in creating a new genus for *striolatus* (with inclusion of the closely-related "*Pristobrycon*" forms that also lack pre-anal spine and ectopterygoid teeth). Older names used for *P. striolatus* include *Serrasalmus* and *Pygocentrus*, neither of which is available. Differences in dentition and other anatomical characters argue against lumping *striolatus* into a single genus together with one or both of the monotypic genera *Pygopristis* Müller & Troschel 1844, and *Catoprion* Müller & Troschel 1844. Ultimately, a study of all *Pristobrycon* species that combines both morphological and genetic analyses will be required to provide final determination on their relationships and true generic identity.

Biogeography and ecophenotypic variation. Because precise information on piranha distributions is lacking, our understanding of piranha biogeography is imperfect. Jégu (1992) presented data on the distribution patterns of certain Serrasalmid taxa and attempted to relate current distributions of selected serrasalmids with glacial and interglacial events of the Quaternary. However, his most detailed analyses involved certain non-piranha taxa (e.g., *Acnodon* and *Mylesinus*). Based on the fossil record and the proposed phylogenetic relationships among serrasalmid genera (Lundberg *et al.* 1986; Fig. 1A), Nico (1991) speculated that no less than a proto-piranha existed before the start of the Pleistocene. If this "first piranha" entered the Quaternary unchanged, subsequent radiation might have been in the form of adaptive responses to the dramatic changes wrought by glacial events (Nico 1991). Our estimate of the average time of the initial radiation based on the control region sequence (Table 3) is consistent with a Plio-Pleistocene origin of this group and may help explain that lack of differentiation among some terminal groups.

The Orinoco and Amazon basins contain the majority of piranha species. Moreover, there is geologic and biologic evidence that the two basins periodically have had very close associations in the past (including as recently as the Late Pleistocene-Holocene), and it has been hypothesized that tectonic events shifted the primary outlet of the central Amazon region a number of times between the north and east (Frailey *et al.* 1988). In more recent times the Orinoco and Amazon were distinct basins and, conceivably, their separate piranha assemblages may have radiated independently to some extent. Today, the two basins are connected by the Casiquiare, a natural waterway flowing southward from the upper Orinoco into the upper Negro.

The role of the Casiquiare in South American fish biogeography is intriguing because the channel is permanent and large, thereby permitting exchange of fishes between the Orinoco and Negro-Amazon. However, the significance of this natural waterway in dispersing piranhas and other fishes is unknown. For example, it is not known which, if any, of the piranha species currently widespread (i.e., occurring in both the Orinoco and Amazon) originated in the Orinoco as opposed to the Amazon. Our data for *S. manueli*, with a paraphyletic Orinoco population giving rise to a primarily Amazonian crown group suggest the direction was Orinoco to Amazon for this species. In any case, any possible reshuffling of piranha distributions by the Casiquiare is likely to have occurred quite recently because the Casiquiare connection to the Orinoco is reportedly very recent (see Stern 1970). Hydrologically, it represents a stream capture in progress that, if gone unchecked, will ultimately lead to the takeover of a large portion of the upper Orinoco by the Negro (Stern 1970; Sternberg 1975). Exactly when the initial connection was formed is uncertain. Some have hypothesized that native Amerindians began the process by cutting a small and fairly short channel over the low area for their canoes to avoid having to portage when crossing from the Orinoco to the Negro system (Raffles & Winkler-Prins 2003; but see Sternberg 1975). In any case, over time, the force of the Orinoco current has increasingly enlarged the uppermost end of the Casiquiare.

Similar to most other piranha species, the precise boundaries of the native distributions of *S. gouldingi*, *S. manueli*, and *Serrasalmus* sp. "A" are not fully known. Consequently, any biogeographic discussion is highly speculative. Based on limited collecting by us and others, we know that both *S. gouldingi* and *S. manueli* are common to the Casiquiare drainage. We initially believed their overall ranges differed significantly, with *S. manueli* in the Orinoco (i.e., a northern species) and *S. gouldingi* in the Negro (i.e., a southern species). Although distribution information is still incomplete, recent literature and museum records (some unconfirmed) indicate *S. manueli* has a broader distribution than previously thought, and includes various major tributaries in the middle and upper Orinoco and the Casiquiare system, as well as a large portion of the Negro mainstem (Fig. 3). There are museum records suggesting its occurrence as far north as the San Bartolo and Aguaro rivers in the Venezuelan Llanos, but two specimens from the region that we examined were found to be incorrectly identified and clearly not *S. manueli*. The Aguaro "*S. manueli*" record is listed by Machado-Allison and Fink (1996:147) although the site is not included in their distribution map for the species. Considering its southern distribution, in addition to the Negro, there is evidence that *S. manueli* occurs in other parts of the Amazon. During a 1986 visit to the Museu de Zoologia of the Universidade de Sao Paulo, one of us (LGN) photographed preserved piranhas (many labeled simply as *Serrasalmus* sp.). We recently re-examined the photographs and realized three adult specimens (MZUSP 15771, 20290, and 25587) from the Tapajos and Trombetas river drainages (Amazon Basin) likely represent *S. manueli* or a closely-related form.

Serrasalmus gouldingi ranges widely in the Negro River, from above its confluence with the Casiquiare downstream to at least as far as the Archipelago das Anavilhanas in the lower Negro (Fig. 3) and probably to its mouth. The species is widely distributed in the mainstem Casiquiare and certain tributaries (e.g., Pasimoni or Pacimoni River). In the Amazon Basin outside the Negro, the only record known to us is that of a single *S. gouldingi* taken from Lago Amana in the lower Japurá River. According to W.G.R. Crampton (*pers. comm.*), the Amana is a blackwater lake with typical blackwater fish fauna. In their original description of *S. gouldingi*, Fink and Machado-Allison (1992) did not include any records from the Orinoco basin. Similarly, a follow-up publication on Venezuelan piranhas indicated *S. gouldingi* is absent from the Orinoco (Machado-Allison & Fink 1996). However, recent publications list the species as occurring in both the Amazon and Orinoco basins (Jégu 2003; Lasso *et al.* 2004). The authors do not provide details and the information may be based on incorrect identifications. We are unaware of any confirmed records of *S. gouldingi* in the Orinoco, although its presence in the Casiquiare suggests the species could freely move north into the basin.

Serrasalmus sp. "A" is relatively widespread in the upper Orinoco. Nico (1991) reported that it (under the name *Serrasalmus* cf. *eigenmanni*) was the third most common piranha in his upper-Orinoco samples. Based largely on the collections of LGN, it appears *Serrasalmus* sp. "A" inhabits primarily clearwater systems or whitewater systems with relatively low sediment loads, including the Orinoco tributaries Mavaca, Ocamo, Padamo-Matacuni, and Ventuari rivers. Its presence in the Casiquiare drainage is uncertain, but would seem likely given the species' occurrence in nearby Orinoco sites. *S. gouldingi*, which is similar in appearance, seems to be restricted, or nearly so, to blackwater habitats.

Ecophenotypic variation in piranha colors. A wide range of neotropical fishes occurring in black- or tannin-stained waters tend to be very darkly colored whereas in white- or muddy waters individuals are much lighter (Araujo-Lima & Goulding 1997; L. G. Nico, pers. obs.). Such differences are often much greater than the subtle color differences used by some ichthyologists to differentiate purported new species. Consequently, we suspect the influence of water type on phenotype (i.e., intensity and pattern of pigmentation) has contributed, on occasion, to erroneous new species descriptions. Tropical South American rivers are generally classified into one of three main types, according to their color and clarity: clear, white, or black. The scheme is by no means perfect because individual rivers may change seasonally or appear to be a mix of more than one type. *Serrasalmus manueli* and *S. gouldingi* are most commonly reported from blackwater systems, although there are exceptions. Fernández-Yépez and Ramirez (1967) based their description of *S. manueli* solely on specimens captured in the Parguaza River (Orinoco Basin, Venezuela; Fig. 4), reporting that the species

occurred in clear water. *Serrasalmus manueli* also is relatively abundant in the nearby Cinaruco River, an Orinoco tributary whose waters, depending on season, may appear as a mix of black and white water types, or sometimes clear (but tinted green from algae) (LGN, pers. obs.). The mainstem Orinoco in its upper reaches has little or no tannin and a relatively low sediment load, possibly best described as clear water.

Conclusions

Piranha systematics are undergoing major revision and much remains to be done. For example, a functional key to identify many juvenile and adult piranhas is lacking. In spite of the hurdles remaining, we are confident that continued and more detailed genetic analyses combined with further scrutiny of morphological characters will ultimately produce a clear picture of piranha and serrasalmid phylogenetic relationships. Progress will require inclusion of other *Serrasalmus* and all or most of the remaining *Pristobrycon* species. Of particular importance relative to the piranha clade is the need to determine if genetic analysis consistently separates all *Pristobrycon* species with pre-anal spines from those without and if, as has so far been shown with *P. striolatus*, those without a pre-anal spine are separate from the clade consisting of piranhas with pre-anal spines. Given the continued confusion and complexity, the work remaining is still substantial. Future studies of serrasalmid phylogeny should include: 1) combined genetic data, including additional unlinked loci and anatomical analysis of new material and reanalysis of older specimens, and 2) careful documentation of specimens examined, including vouchering of all material and establishment of photographic archives of specimens studied.

Acknowledgments

This research was carried out as an undergraduate senior project by BF, supported by the NSF Undergraduate Mentoring in Environmental Biology program. Activities in South America, including field collecting and museum visits, were supported in part by grants to LGN from the Andrew W. Mellon Foundation (Amazon Research and Training Program, University of Florida) and various National Geographic Society grants. We are especially grateful to Kirk O. Winemiller for inviting LGN and HLJ to participate in a 1999 expedition to the Casiquiare. For laboratory, field, and logistic help in South America, LGN thanks the administrators, researchers, technicians, and field workers associated with the Universidad Nacional de los Llanos Occidentales "Ezequiel Zamora" and Museo de Zoología (UNELLEZ-MCNG) and Corporación Venezolana Guayana-Técnica Minera (CVG-TECMIN). We are indebted to Anthony B. Falsetti and Nicole DeMers for help with x-rays. Various museum personnel and university researchers assisted by providing specimens loans, photographs, or other information. For their help we thank: Carlos Lasso and Oscar M. Lasso-Alcalá (Museo de Historia Natural La Salle), Robert H. Robins (Florida Museum of Natural History), William F. Fink (University of Michigan), Donald C. Taphorn (UNELLEZ-MCNG), Mike Retzer (Illinois Natural History Survey), Frank Pezold (NLU), and Flávio C. T. Lima (MZUSP). We are grateful to William Smith-Vaniz and Timothy Rawlings for critically reviewing various drafts of the manuscript. Lisa Jelks kindly edited the final draft. Lastly, we thank the Zootaxa editors for their time and assistance.

References

Agostinho, C.S., Agostinho, A.A., Marques, E.E. & Bini, L.M. (1997) Abiotic factors influencing piranha attacks on netted fish in the upper Parana River, Brazil. *North American Journal of Fisheries Management*, 17, 712–718.

Araujo-Lima, C. & Goulding, M. (1997) *So Fruitful a Fish: Ecology, Conservation, and Aquaculture of the Amazon's Tambaqui*. Columbia University Press, New York, 191 pp.

Arratia, G. & Cione, L.A. (1996) The fossil record of fossil fishes of Southern South America. *In*: Arratia, G. (Ed.), *Con-*

tributions of Southern South America to Vertebrate Paleontology. Special volume of the Münchner Geowissenschaftliche Abhandlungen 30(A), pp. 9–72.
Benson, G. (1999) Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acids Research*, 27, 573–580.
Braga, R.A. (1975) *Ecologia e Etologia de Piranhas no Nordeste do Brasil (Pisces–Serrasalmus Lacépède, 1803)*. Banco do Nordeste do Brasil, Fortaleza, Ceará, Brazil, 268 pp.
Braun, E.L. & Kimball, R.T. (2001) Polytomies, the power of phylogenetic inference, and the stochastic nature of molecular evolution: a comment on Walsh *et al*. (1999). *Evolution*, 55, 1261–1263.
Calcagnotto, D., Schaefer, S.A. & DeSalle, R. (2005) Relationships among characiform fishes inferred from analysis of nuclear and mitochondrial gene sequences. *Molecular Phylogenetics and Evolution*, 36, 135–153.
Castagnoli, N. (2000) Brazil fish culture. *In*: Stickney, R.R. (Ed.), *Encyclopedia of Aquaculture*. John Wiley and Sons, New York, pp. 123–128.
Cestari, M.M. & Galetti Jr., P.M. (1992) Chromosome evolution in the genus *Serrasalmus* and cytotaxonomic considerations about Serrasalminae (Characidae, Pisces). *Revista Brasileira de Genética*, 15, 555–567.
Donaldson, K.A. & Wilson, R. R. (1999) Amphi-panamic geminates of snook (Percoidei: Centropomidae) provide a calibration of the divergence rate in the mitochondrial DNA control region of fishes. *Molecular Phylogenetics and Evolution*, 13, 208–213.
Eigenmann, C.H. (1915) The Serrasalminae and Mylinae. *Annals of the Carnegie Museum,* 9(3–4), 226–272.
Fernández-Yepez, A. & Ramírez, M.V. (1967) *Los caribes (serrasalmidos) de Venezuela y las pesqueras*. Trabajos anexos a la Comisión Contribuciones al Tema, Ier Foro Internacional sobre Planificación y Desarrollo Pesquero, Caracas, Venezuela, 25 pp., figures. (mimeo).
Fink, W.L. (1988) Evolutionary biology of piranhas. *In:* Dalrymple, G.H., Loftus, W.F. & Bernardino, F.S. (Eds.), *Wildlife in the Everglades and Latin American Wetlands*. Abstracts on the Proceedings of the First Everglades National Park Symposium, held February 25–March 1, 1985. Florida International University, Miami, Florida, pp. 13–14.
Fink, W.L. (1989) Ontogeny and phylogeny of shape change and diet in the South American fishes called piranhas. *In*: David, B., Dommergues, J.-L., Chaline, J. & Laurin, B. (Eds.), *Ontogenese et Évolution*. *Geobios*, *mémoire special*, No. 12, 167–172.
Fink, W.L. (1993) Revision of the piranha genus *Pygocentrus* (Teleostei, Characiformes). *Copeia*, 1993, 665–687.
Fink, W.L. & Machado-Allison, A. (1992) Three new species of piranhas from Brazil and Venezuela (Teleostei: Characiformes). *Ichthyological Exploration Freshwaters*, 3, 55–71.
Fink, W.L. & Zelditch, M.L. (1995) Phylogenetic analysis of ontogenetic shape transformations: a reassessment of the piranha genus *Pygocentrus* (Teleostei). *Systematic Biology*, 44, 343–360.
Fink, W.L. & Zelditch, M.L. (1997) Shape analysis and taxonomic status of the *Pygocentrus* piranhas (Ostariophysi, Characiformes) from the Paraguay and Paraná river basins of South America. *Copeia*, 1997, 179–182.
Frailey, C.D., Lavina, E.L., Rancy, A. & de Souza–Filho, J.P. (1988) A proposed Pleistocene/Holocene lake in the Amazon basin and its significance to Amazonian geology and biogeography. *Acta Amazonica*, 18, 119–143.
Gaviria, J.I., Nirchio, M., Granado, A. & Estrada, A. (2005). Karyotype and nucleolar organizer regions of *Pygocentrus cariba* (Serrasalminae) from Caricara del Orinoco, Venezuela. *Interciencia*, 30, 44–47.
Géry, J. (1972) Poissons characoides des Guyanes II. Famille des Serrasalmidae. *Zoologische Verhandelingen Leiden*, 122, 134–248.
Géry, J. (1976) Les genres de Serrasalminae (Pisces–Characoidei). *Bulletin Zoologisch Museum Universiteit van Amsterdam*, 5, 47–54.
Géry, J. (1977) *Characoids of the World*. Tropical Fish Hobbyist Publications, Neptune City, New Jersey, 672 pp.
Goldman, N. & Whelan, S. (2000) Statistical tests of gamma-distributed rate heterogeneity in models of sequence evolution in phylogenetics. *Molecular Biology and Evolution*, 17, 975–978.
Gosline, W.A. (1951) Notes on the characid fishes of the subfamily Serrasalminae. *Proceedings of the California Academy of Sciences*, 27, 17–64.
Goulding, M. (1980) *The Fishes and the Forest: Explorations in Amazonian Natural History*. University of California Press, Berkeley, 280 pp.
Goulding, M., Carvalho, M.L. & Ferreira, E.G. (1988) *Rio Negro, Rich Life in Poor Water*. SPB Academic Publishing, The Hague, The Netherlands, 200 pp.
Haddad, V., Jr. & Sazima, I. (2003) Piranha attacks on humans in southeast Brazil: epidemiology, natural history, and clinical treatment, with description of a bite outbreak. *Wilderness and Environmental Medicine*, 14, 249–254.
Hubert, N., Duponchelle, F., Nuñez, J., Rivera, R. & Renno, J.-F. (2006) Evidence of reproductive isolation among closely related sympatric species of *Serrasalmus* (Ostariophysii, Characidae) from the Upper Madeira River, Amazon, Bolivia. *Journal of Fish Biology*, 69, 31–51.
Jégu, M. (1992) Variations du niveau marin et distribution des poisons d'eau douce en Amazonie Orientale. *In*: Prost, M.T. (Ed.), *Evolution des littoraux de Guyane et de la zone caraïbe méridionale pendant le quaternaire*. ORSTOM, Paris, pp. 281–297.
Jégu, M. (2003) Subfamily Serrasalminae (pacus and piranhas). *In*: Reis, R.E., Kullander, S.O. & Ferraris, C.J., Jr.

(Eds.), *Check list of Freshwater Fishes of South and Central America*. Edipucrs, Porto Alegre, Brazil, pp. 182–196.
Jégu, M. & Dos Santos, G.M. (1988) Le genre *Serrasalmus* (Pisces, Serrasalmidae) dans le bas Tocantins (Brésil, Parà), avec la description d'une espèce nouvelle, *S. geryi*, du basin Araguaia–Tocantins. *Revue d'Hydrobiologie Tropicale*, 21, 239–274.
Jégu, M. & Dos Santos, G.M. (2001) Mise au point à propos de *Serrasalmus spilopleura* Kner, 1858 et réhabilitation de *S. maculatus* Kner, 1858. *Cybium*, 25, 119–143.
Jégu, M., Leão, E.L.M. & Dos Santos, G.M. (1991) *Serrasalmus compressus*, une espece nouvelle du Rio Madeira, Amazonie (Pisces: Serrasalmidae). *Ichthyological Exploration of Freshwaters*, 2, 97–108.
Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F.X. & Wilson, A.C. (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Science of the United States of America*, 86, 6196–6200.
Lasso, C.A., Mojica, J.I., Usma, J.S., Maldonado-O., J.A., DoNascimiento, C., Taphorn, D.C., Provenzano, F., Lasso-Alcala, O., Galvis, G., Vasquez, L., Lugo, M., Machado-Allison, A., Royero, R., Suárez, C. & Ortega-Lara, A. (2004). Peces de la cuenca del río Orinoco. Parte I: lista de especies y distribución por subcuencas. *Biota Colombiana* 5 (2), 95–158.
Lovejoy, N.R. 1997. Stingrays, parasites, and neotropical biogeography: a closer look at Brooks *et al.*'s hypotheses concerning the origins of neotropical freshwater rays (Potamotrygonidae). *Systematic Biology*, 46, 218–230.
Ludwig, A., May, B., Debus, L. & Jenneckens, I. (2000) Heteroplasmy in the mtDNA control region of sturgeon (*Acipenser*, *Huso* and *Scaphirhynchus*). *Genetics*, 156, 1933–1947.
Lundberg, J.G., Machado-Allison, A. & Kay, R.F. (1986) Miocene characid fishes from Colombia: evolutionary stasis and extirpation. *Science*, 234, 208–209.
Lundberg, J.G. (1997) Freshwater fishes and their paleobiotic implications. *In*: Kay, R.F., Madden. R.L., Cifelli, R.L. & Flynn, J.J (Eds.), *Vertebrate Paleontology in the Neotropics: The Miocene Fauna of La Venta, Columbia*. Smithsonian Institution Press, Washington, D.C., pp. 67–91.
Lundberg, J.G. (1998) The temporal context for the diversification of neotropical fishes. *In*: Malabarba, L.R., Reis, R.E., Vari, R.P., Lucena, Z.M.S. & Lucena, C.A.S. (Eds.), *Phylogeny and Classification of Neotropical Fishes*. Edipucrs, Porto Alegre, Brazil, pp. 49–68.
Lunt, D.H., Whipple, L.E. & Hyman, B.C. (1998) Mitochondrial DNA variable number tandem repeats (VNTRs): utility and problems in molecular ecology. *Molecular Ecology*, 7, 1441–1455.
Machado-Allison, A. (1982) *Studies on the systematics of the subfamily Serrasalminae (Pisces–Characidae)*. Unpublished Ph.D. dissertation, The George Washington University, Washington D.C.
Machado-Allison, A. (1983) Estudios sobre la sistematica de la subfamilía Serrasalminae (Teleostei, Characidae). Parte 2. Discusión sobre la condición monofilética de la subfamilía. *Acta Biologica Venezuelica*, 11, 145–195.
Machado-Allison, A. (1985) Estudios sobre la sistemática de la subfamilia Serrasalminae. Parte III. Sobre el estatus generico y relaciones filogenéticas de los géneros *Pygopristis*, *Pygocentrus*, *Pristobrycon* y *Serrasalmus* (Teleostei–Characidae–Serrasalminae). *Acta Biologica Venezuelica*, 12, 19–42.
Machado-Allison, A. (2002) Los peces caribes de Venezuela: una aproximación a su estudio taxonómico. *Boletín de la Academia de Ciencias Físicas, Matemáticas y Naturales de Venezuela*, 62(1), 35–88.
Machado-Allison, A. & Fink, W. (1995) *Sinopsis de las especies de la subfamilia Serrasalminae presentes en la cuenca del Orinoco: claves, diagnosis e ilustraciones.* Serie Peces de Venezuela, Universidad Central de Venezuela, Caracas, 90 pp.
Machado-Allison, A. & Fink, W. (1996) *Los Peces Caribes de Venezuela: Diagnosis, Claves, Aspectos Ecologicos y Evolutivos*. Collection Monograph 52, Universidad Central de Venezuela, Caracas, 151 pp.
Machado-Allison, A., Fink, W. & Antonio, M. E. (1989) Revisión del género *Serrasalmus* Lacepede, 1803 y géneros relacionados en Venezuela: I. Notas sobre la morfología y sistematica de *Pristobrycon striolatus* (Steindachner, 1908). *Acta Biologica Venezuelica*, 12, 140–171.
Merckx, A., Jégu, M. & Dos Santos, G.M. (2000). Une nouvelle espèce de *Serrasalmus* (Teleostei: Characidae: Serrasalminae), *S. altispinis* n. sp., décrite du rio Uatumã (Amazonas, Brésil) avec une description complémentaire de *S. rhombeus* (Linnaeus, 1766) du Plateau Guyanais. *Cybium*, 24, 181–121.
Nakayama, C.M., Jégu, M., Porto, J.I.R. & Feldberg, E. (2001) Karyological evidence for a cryptic species of piranha within *Serrasalmus rhombeus* (Characidae, Serrasalminae) in the Amazon. *Copeia*, 2001, 866–869.
Nakayama, C.M., Porto, J.I.R. & Feldberg, E. (2002) A comparative cytogenetic study of five piranha species (*Serrasalmus*, Serrasalminae) from the Amazon basin. *Genetica*, 114, 231–236.
Nico, L.G. (1991) *Trophic ecology of piranhas (Characidae: Serrasalminae) from savanna and forest regions in the Orinoco River basin of Venezuela*. Unpublished Ph.D. dissertation, University of Florida, Gainesville, 210 pp.
Nico, L.G. & Taphorn, D.C. (1986) Those bitin' fish from South America. *Tropical Fish Hobbyist*, 34 (Feb.), 24–27, 30–34, 36, 40–41, 56–57.
Nico, L.G. & Taphorn, D.C. (1988) Food habits of piranhas in the low Llanos of Venezuela. *Biotropica*, 20, 311–321.
Nirchio, M., Granado, A., Ron, E. & Pérez, J.E. (2005). Karyotype and nucleolar organizer regions in *Serrasalmus*

rhombeus (Serrasalminae) from Caricara del Orinoco, Venezuela. *Interciencia*, 27, 676–678.
Norman, J.R. (1929) The South American characid fishes of the subfamily Serrasalmoninae, with a revision of the genus *Serrasalmus* Lacepède. *Proceedings of the Zoological Society of London*, 52 (1928), 781–829.
Oliveira C., Almeida-Toledo L.F., Foresti F., Britski H. & Toledo-Filho S.A. (1988) Chromosome formulae of Neotropical freshwater fishes. *Revista Brasileira de Genética*, 11, 577–624.
Ortí, G., Petry, P., Porto, J.I.R., Jégu, M. & Meyer, A. (1996) Patterns of nucleotide change in mitochondrial ribosomal RNA genes and the phylogeny of piranhas. *Journal of Molecular Evolution*, 42, 169–182.
Ortí, G., Porto, J.I.R. & Sivasundar, A. (2000) *Phylogeny of the Serrasalminae (Characiformes) based on mitochondrial DNA sequences*. Unpublished poster presented at the 80th Annual Meeting of the American Society of Ichthyologists and Herpetologists, La Paz, Baja California Sur, Mexico (14–20 June 2000). Available from http://golab.unl.edu/people/asivasun/index.html (accessed 30 May 2005)
Palumbi, S.R. (1996) Nucleic acids II: The polymerase chain reaction. *In*: Hillis, D.M., Moritz, C. & Mable, B.K. (Eds.), *Molecular Systematics*, *2nd edition*. Sinauer Associates, Sunderland, Massachusetts, pp. 205–247.
Porto, J.I.R., Feldberg, E., Nakayama, C.M., Maia, R.O. & Jégu, M. (1991) Cytotaxonomic analysis in the Serrasalmidae (Ostariophysi, Characiformes). Abstracts of presentations, Seventh International Ichthyology Congress of the European Ichthyological Union, The Hague, The Netherlands. *Bulletin Zoologisch Museum, Universiteit van Amsterdam* (special issue), 66.
Porto, J.I.R., Feldberg E., Nakayama, C. & Falcao, J.N. (1992) A checklist of chromosome numbers and karyotypes of Amazonian freshwater fishes. *Revue d'Hydrobiologie Tropicale*, 25, 287–299.
Posada D. & Crandall, K.A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics*, 14, 817–818.
Raffles, H. & Winkler-Prins, A.M.G.A. (2003) Further reflections on Amazonian environmental history: transformations of rivers and streams. *Latin American Research Review*, 38, 165–187.
Ravago, R.G., Monje, V.D. & Juinio-Menez, M.A. (2002) Length and sequence variability in mitochondrial control region of the Milkfish, *Chanos chanos*. *Marine Biotechnology*, 4, 40–50.
Ray, D.A. & Densmore, L. (2002) The crocodilian mitochondrial control region: General structure, conserved sequences, and evolutionary implications. *Journal of Experimental Zoology*, 294, 334–345.
Reis, R.E. (1998) Systematics, biogeography, and the fossil record of the Callichthyidae: a review of the available data. *In*: Malabarba, L.R., Reis, R.E., Vari, R.P., Lucena, Z.M.S. & Lucena, C.A.S. (Eds.), *Phylogeny and Classification of Neotropical Fishes*. Edipucrs, Porto Alegre, Brazil, pp. 351–374.
Ronquist, F. & Huelsenbeck, J. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19, 1572–1574.
Rubilar, A. (1994) Diversidad ictiológica en depósitos continentales miocenos de la Formación Cura–Mallín, Chile (37–39°S): implicancias paleogeográficas. *Revista Geológica de Chile*, 21, 3–29.
Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A. & Allard, R.W. (1984) Ribosomal DNA spacer length in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Science of the United States of America*, 81, 8014–8018.
Sazima, I. & Andrade–Guimaraes, S. (1987) Scavenging on human corpses as a source for stories about man–eating piranhas. *Environmental Biology of Fishes*, 20, 75–77.
Sazima, I. & Machado, F.A. (1990) Underwater observations of piranhas in western Brazil. *Environmental Biology of Fishes*, 28, 17–31.
Sivasundar, A., Bermingham, E. & Ortí , G. (2001) Population structure of migratory freshwater fishes (*Prochilodus*: Characiformes) in major South American rivers. *Molecular Ecology*, 10, 407–418.
Slowinski, J.B. (2001) Molecular polytomies. *Molecular Phylogenetics and Evolution*, 19, 114–120.
Stern, K.M. (1970) Der Casiquiare–Kanal, einst und jetzt. *Amazoniana*, 2, 401–416.
Sternberg, H.O. (1975) The Amazon River of Brazil. *Geographische Zeitschrift*, 40, 1–74.
Swofford, D.L. (2002) *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Sinauer Associates, Sunderland, Massachusetts.
Thompson, J.D., Gibson T.J., Plewniak F., Jeanmougin F. & Higgins D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25(24), 4876–82.
Van Every, L.R. & Kritsky, D.C. (1992) Neotropical Monogenoidea. 18 *Anacanthorus* Mizelle and Price, 1965 (Dactylogyridae, Anacanthorinae) of piranha (Characoidea, Serrasalmindae) from the Central Amazon, their phylogeny, and aspects of host–parasite coevolution. *Journal of Helminthological Society of Washington*, 59, 52–75.
Walsh, H.E. & Friesen, V.L. (2001) Power and stochasticity in the resolution of soft polytomies: A reply to Braun *et al*. *Evolution*, 55, 1264–1266.
Walsh, H.E., Kidd, M.G., Moum, T. & Friesen, V.L. (1999) Polytomies and the power of phylogenetic inference. *Evolution*, 53, 932–937.
Winemiller, K.O. (1989) Ontogenetic diet shifts and resource partitioning among piscivorous fishes in the Venezuelan llanos. *Environmental Biology of Fishes*, 26, 177–199.

Appendix 1

Photographs of live and preserved specimens of selected serrasalmine taxa used as source material for genetic analyses in this study. Specimen numbers (1–31) refer to material listed in Table 1 and presented in distribution map (Figure 4) and cladograms (Figures 7 and 8). Photographs of live or fresh material are indicated by "L". Photographs in boxes indicate specimens from same site as the genetic specimens. Hyaline (clear) portions of fins show background color (typically blue or gray). Specimens are proportional to scale bar in millimeters. All photographs by Howard Jelks and Leo Nico unless otherwise indicated.

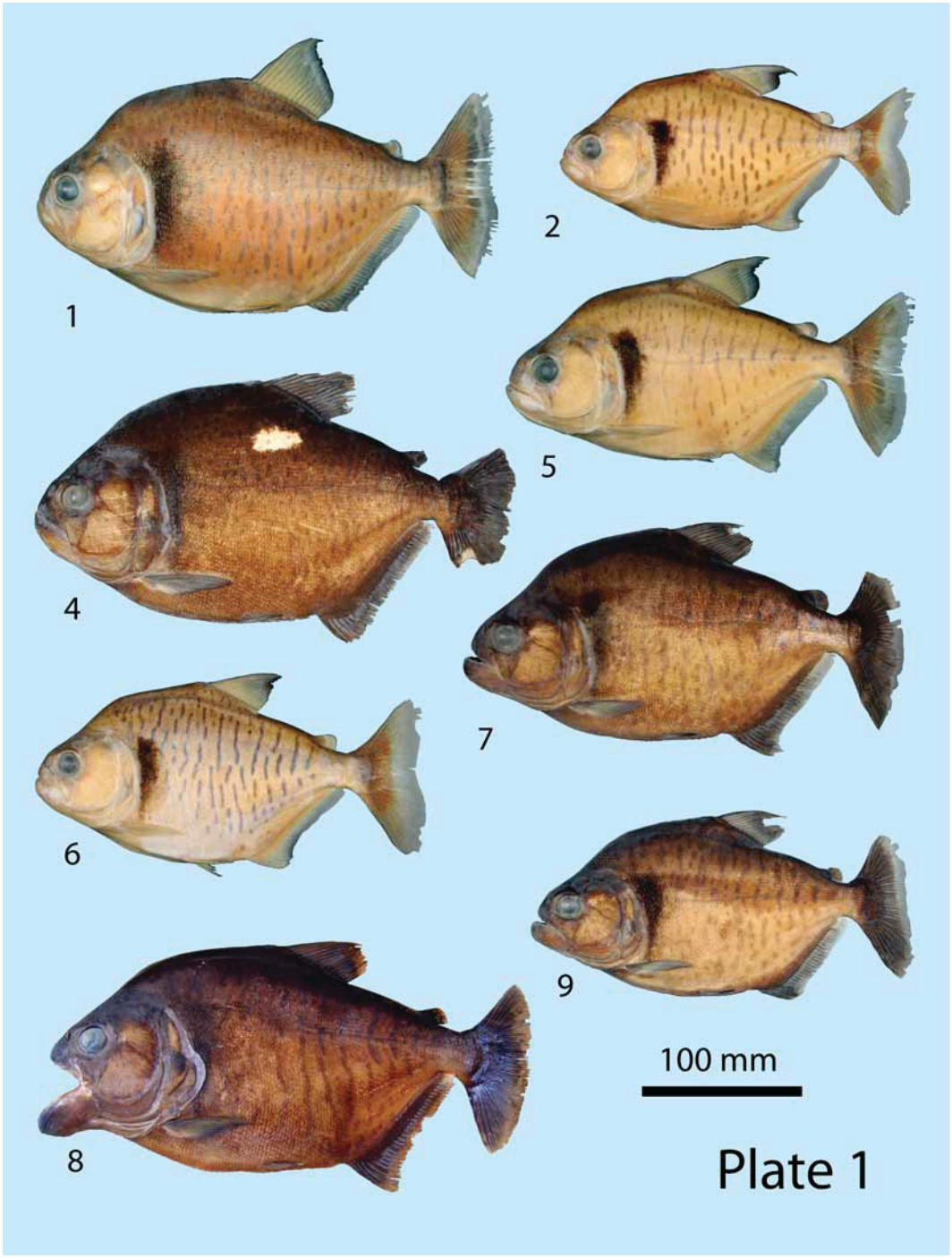

PLATE 1. *Serrasalmus manueli* (1–9). (No photograph or voucher available for specimen 3.) (Photograph of 8-*S. manueli* by Frank Pezold.)

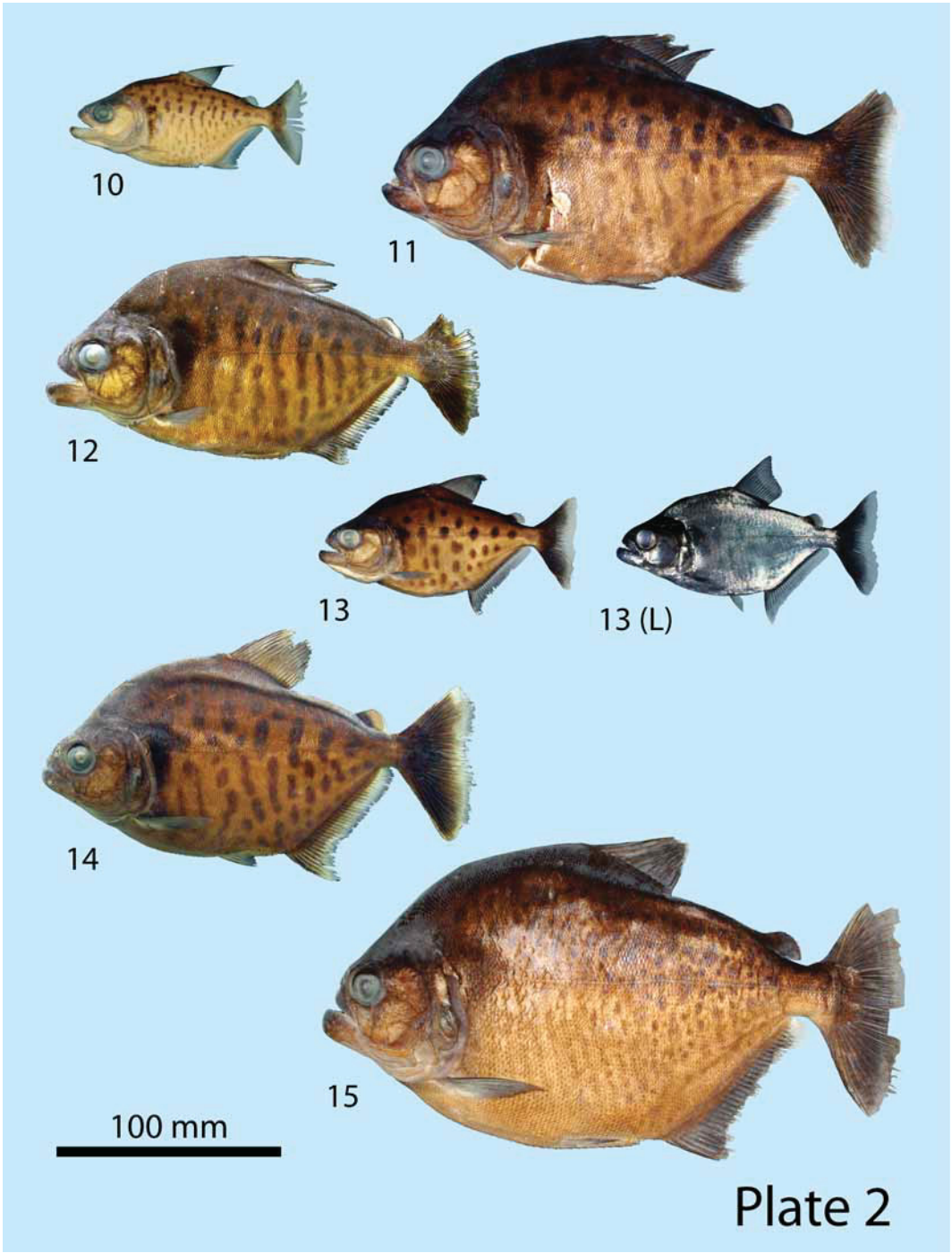

PLATE 2. *Serrasalmus manueli* (10) and *S. gouldingi* (11–15). (Photographs of *S. gouldingi* specimens 12 and 14 by Donald Taphorn.)

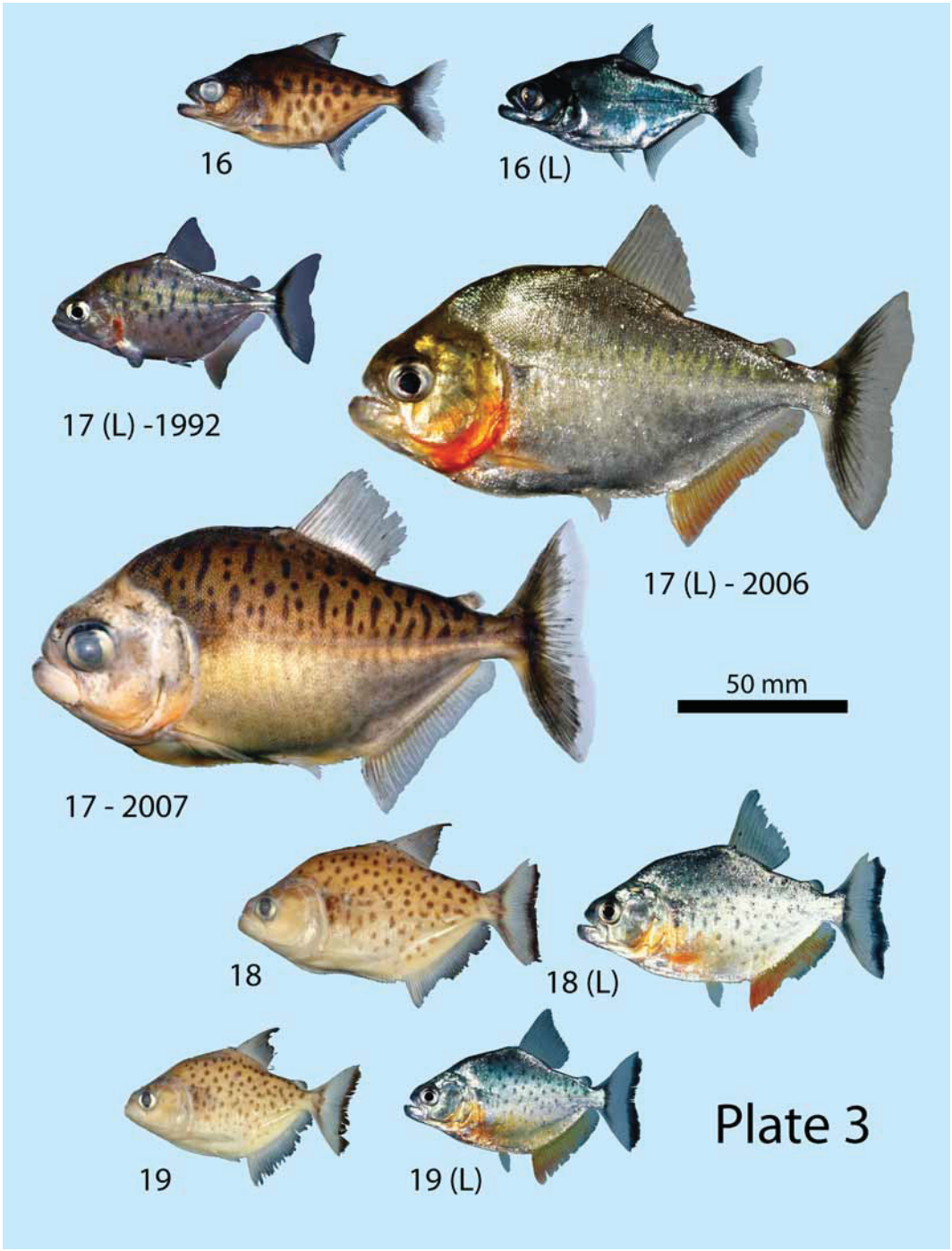

PLATE 3. *Serrasalmus gouldingi* (16), *Serrasalmus* sp. A (17), and *S. medinai* (18–19). Images of *Serrasalmus* sp. "A" are of specimen 17 (originally captured in 1991) at different ages, including the same fish live in captivity photographed in 1993 (as juvenile about 2+ years old), 2006, and after preservation in 2007.

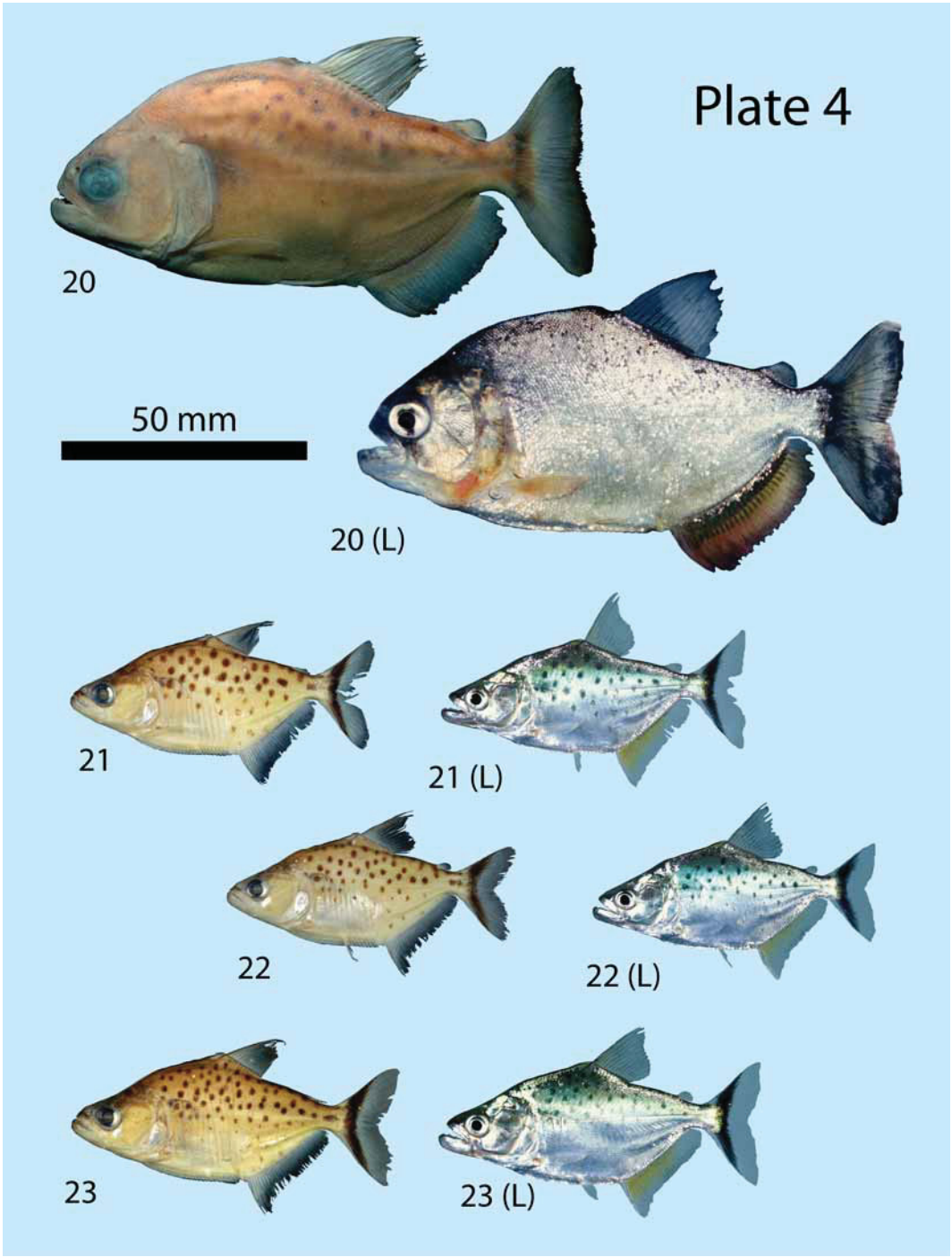

PLATE 4. *Serrasalmus medinai* (20) and *S. irritans* (21–23). (Photograph of live 20-*S. medinai* by Noel Burkhead.)

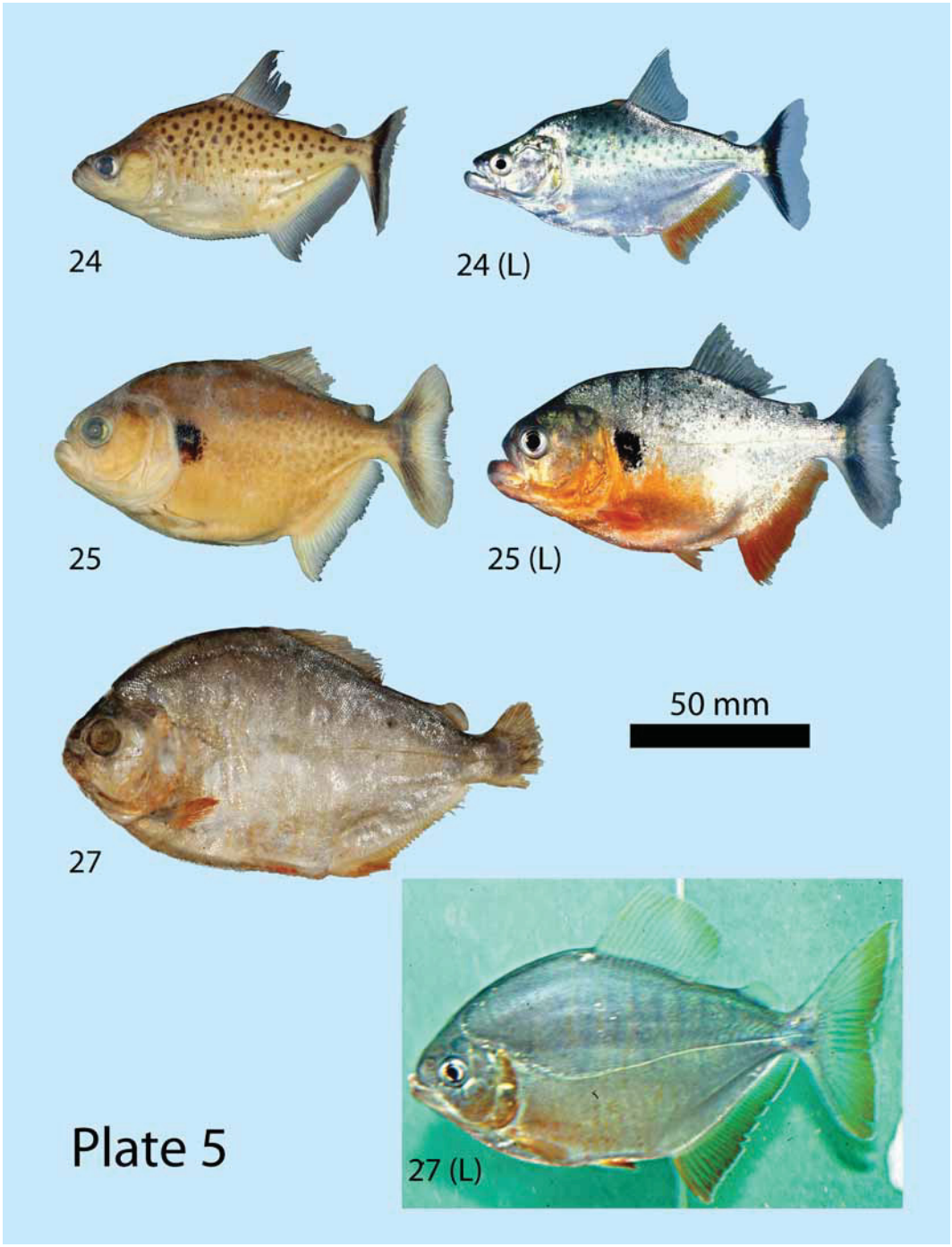

PLATE 5. *Serrasalmus irritans* (24), *Pygocentrus cariba* (25), and *Pygopristis denticulatus* (27). (No photograph or voucher available for specimen 26.)

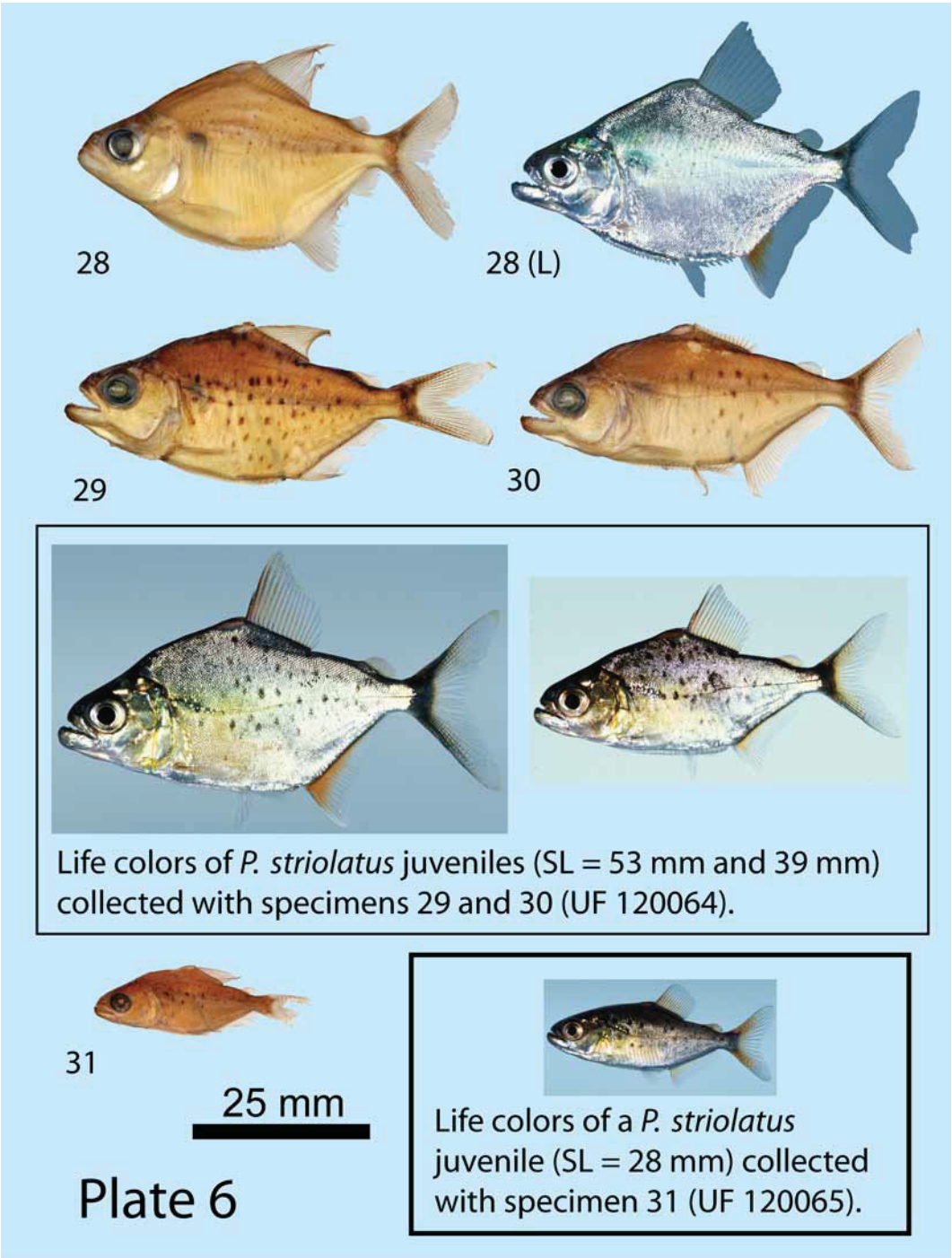

PLATE 6. *Pristobrycon striolatus* (28–31) and additional small juvenile specimens collected with genetic vouchers showing life colors.

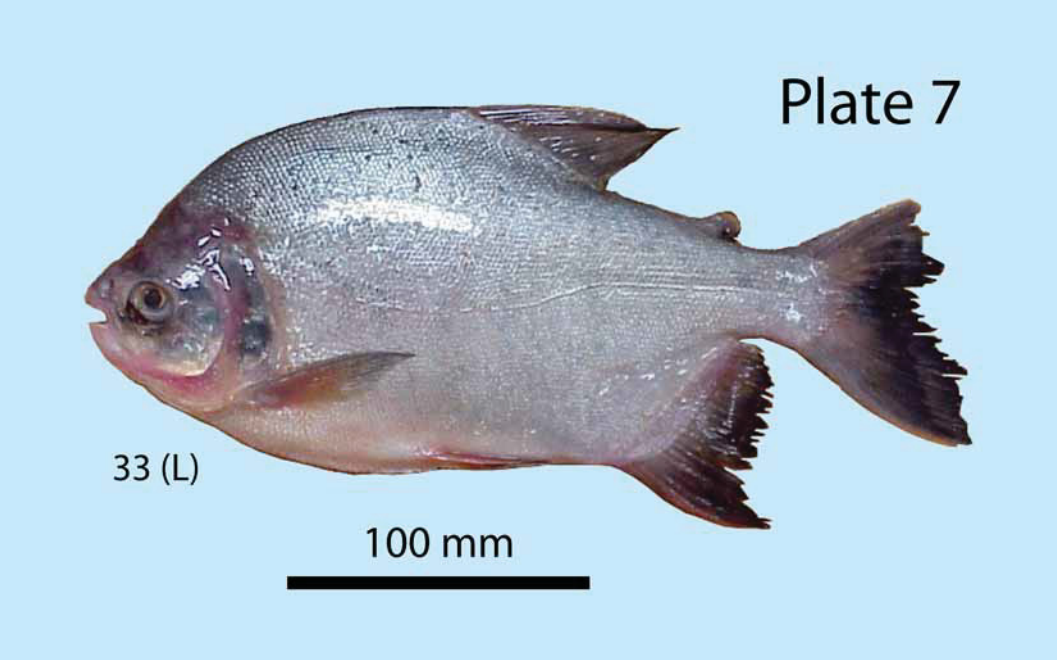

PLATE 7. *Piaractus brachypomus* (33) (Photograph provided by Robert Lea). (No photograph or voucher available for specimen 32.)

Appendix 2. GenBank sequences used in this study.

Species ID	GenBank Accession Numbers			Locality	Citation
	Control region	16S	12S		
Serrasalmus spilopleura	AF283948	U33592	U33560	Rio Uruguay, Salto Grande, Argentina	Ortí *et al.* (1996)
Serrasalmus sp.		U33593	U33561	Rio Negro-Solimoes, AM, Brazil	Ortí *et al.* (1996)
Serrasalmus compressus		U33594	U33562	Rio Solimoes, Ilha da Marchantaria, AM, Brazil	Ortí *et al.* (1996)
Serrasalmus sp. 218		AF283935	AF283914		
Serrasalmus sp. 219		AF283936	AF283915		
Serrasalmus rhombeus		AF283937	AF283920		
Serrasalmus rhombeus str. 222	AF283952	AF281941	AF283916		
Serrasalmus compressus str. 2241		AF283938	AF283917		
Serrasalmus compressus		AF283939	AF283918		
Serrasalmus humeralis		AF283940	AF283919		
Serrasalmus sp. 220	AF283951				
Serrasalmus manueli str. p18	AF283950	AF283942	AF283921		
Serrasalmus gouldingi str. P17	AF283944	AF283943	AF283922		
Serrasalmus eigenmanni	AF283946				
Pygocentrus cariba	AF283954				
Pygocentrus nattereri str. INPA10143	AF283953	U33590	U33558	Rio Solimoes, Ilha da Marchantaria, AM, Brazil	Ortí *et al.* (1996)
*Pygocentrus nattereri*str. USNM325686		U33591	U33559	Rio Uruguay, Salto Grande, Argentina	Ortí *et al.* (1996)
Pygopristis denticulatus str. p4	AF284464				
Pristobrycon sp. 256	AF283949				
Pristobrycon serralatus	AF283945				
Pristobrycon sp. 224	AF283947	U33595	U33563	Rio Solimoes, Ilha da Marchantaria, AM, Brazil	Ortí *et al.* (1996)
Pristobrycon striolatus str. 225	AF284463	U33596	U33597	Rio Pitinga, UHE do Pitinga, AM, Brazil	Ortí *et al.* (1996)
Pristobrycon striolatus str. 226		U33598	U33564	Rio Pitinga, UHE do Pitinga, AM, Brazil	Ortí *et al.* (1996)
Catoprion mento str. 80	AF284462	U33599	U33565	commercial source, locality unknown	Ortí *et al.* (1996)
Metynnis sp.		U33600	U33566	commercial source, locality unknown	Ortí *et al.* (1996)
Metynnis cf. Mola		U33601	U33567	Rio Miranda, Pantanal Matogrossense, Campo Grande, MS, Brazil	Ortí *et al.* (1996)

Metynnis hypsauchen	AF283957	AF283934	AF283913	
Metynnis sp. p31	AF283955			
Metynnis sp. p32	AF283956	AF283933	AF283912	
Ossubtus xinguense str. 253	AF284461			
Acnodon normani str. 243	AF284460			
Myleus rhombeus	AF283976			
Myleus sp. p51	AF283975			
Myleus sp. p49	AF283974			
Myleus pacu str. p33	AF283970			
Myleus pacu str. 238	AF283969			Rio Pitinga, Cachoeira 40 Ilas, AM, Brazil
Myleus schomburgkii	AF283968			Rio Pitinga, Cachoeira 40 Ilas, AM, Brazil
Myleus ternetzi	AF283967			
Myleus tiete	AF283966			Rio Miranda, Pantanal Matogrossense, Campo Grande, MS, Brazil
Myleus rubripinnis	AF283965			commercial source, locality unknown
Myleus asterias	AF283964			R. Pitinga, UHE do Pitinga, AM, Brazil
Mylesinus paucisquamatus	AF283973			
Mylesinus paraschomburgkii	AF283971			R. Pitinga, Cachoeira 40 Ilas, AM, Brazil
Tometes sp. 246	AF283972			
Colossoma macropomum	AF283963			R. Solimoes, Ilha da Marchantaria, AM, Brazil
Mylossoma paraguayensis	AF283962			R. Miranda, Pantanal Matogrossense, Campo Grande, MS, Brazil
Mylossoma duriventri	AF283961			R. Solimoes, Ilha da Marchantaria, AM, Brazil
Piaractus brachipomus str. 45	AF283960			commercial source, locality unknown
Piaractus brachipomus str. 200	AF283958			R. Solimoes, Ilha da Marchantaria, AM, Brazil
Piaractus mesopotamicus	AF283959			R. Miranda, Pantanal Matogrossense, Campo Grande, MS, Brazil